

REMARKS

Applicants have amended Claim 7, support for which is found on pages 6-7 of the specification. Applicants have canceled Claims 8 and 10. Claims 7 and 18-20 remain pending in this application.

§112, paragraph 1, Rejection

Examiner rejected claims 7, 8, 10, 18-20 in view of the first paragraph of 35 USC §112. Applicants traverse this rejection because *in vitro* phase II activity of *Echinacea purpurea* fractions are predictive of *in vivo* phase II activity. In support of its position, Applicants submit in Exhibit A a Declaration under 37 C.F.R. §1.132.

§112, paragraph 2, Rejection

Examiner rejected claims 7, 8, 10, 18-20 in view of the second paragraph of 35 U.S.C. §112. Applicants have amended Claim 1 to recite who the composition is being administered to. As such, paragraph two of §112 is believed to be satisfied.

§102(e) Rejection

Examiner rejected Claims 7, 8, 10, 18-20 under 35 U.S.C. §102(e) as being anticipated by Mitscher et al. or Raskin et al. Applicants respectfully traverse this rejection. Mitscher's earliest priority date is May 9, 2000. Applicants had possession of their invention prior to May 9, 2000 and submit in Exhibit B a Declaration under 37 C.F.R. §1.131 establishing a prior date of conception and reduction to practice. Accordingly, Mitscher is not proper §102(e) prior art.

Furthermore, Applicants submit that Raskin does not disclose all the elements of amended Claim 7. Specifically, Raskin does not disclose chloroform root fractions or acidic chloroform aerial fractions of *Echinacea purpurea*. Rather, Raskin discloses chloroform as one solvent to remove cuticular material from plant leaves (Raskin para 15). Nowhere in Raskin is there mention of adjusting the pH of the cuticular material removed by the chloroform or using chloroform on root extracts. As such, Applicants request removal of this rejection.

§103(e) Rejection over Raskin et al. or Mitscher et al.

Examiner rejected Claims 7, 8, 10, and 18-20 under 35 U.S.C. §103(a) over Mitscher et al or Raskin et al. Applicants respectfully traverse this rejection. As

explained above, because Mitscher is not proper §102(e) prior art, it cannot be used as a reference under 103(a).

With respect to Raskin, Applicants respectfully submit that Examiner has not established a proper *prima facie* case of obviousness because there is no incentive to modify Raskin and no reasonable expectation of success that the modified Raskin extract would result in the claimed method - inducing phase II enzymes with chloroform soluble *Echinacea purpurea* fractions. MPEP §2142 states that to establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, whether in the references themselves or in the knowledge generally available to one of ordinary skill in the art to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. MPEP §2142. Raskin does not teach or suggest that chloroform root fractions or acidic chloroform aerial fractions of *Echinacea purpurea* should be administered to subjects to induce phase II activity. Rather, Raskin teaches that chloroform can be used to remove the cuticular material on plant leaves to obtain compounds such as lipids, wax, cutin, and protein therein (Raskin para. 15). Cuticular material is not even found on *Echinacea* roots so one of ordinary skill in the art would not use the teachings of Raskin to obtain phase II induction compounds from roots. Further, there is no mention or suggestion of adjusting the pH of Raskin's cuticular leaf extract.

Even if Raskin provided incentive to use chloroform fractions of root material or acidic chloroform fractions of aerial material, using chloroform soluble extracts of *Echinacea purpurea* is not taught or suggested. One of Raskin's objectives is to identify plants having therapeutic activity such as anti-microbial and anti-cancer activity (Raskin para. 16). Raskin states that inhibition of the growth of the suspension contacted with the microorganism is indicative of an agent in the suspension having therapeutic activity (Raskin, para. 70). Raskin then tested several plant materials for their activity to inhibit microorganisms and *Echinacea purpurea* for its anti-microbial activity. Specifically,

Raskin lists *Echinacea purpurea* in a single instance - as one of several plant extracts that have no detectable anti-microbial activity (Raskin, para. 169 and page 29). This suggests that *Echinacea purpurea* is not an efficacious anti-microbial material for accomplishing Raskin's objectives. As such, one of ordinary skill in the art would not consider using Raskin's *Echinacea purpurea* material to successfully obtain an anti-microbial compound let alone phase II induction. There is no reasonable expectation of succession modifying Raskin's extract to obtain the claimed invention. Therefore, Applicants respectfully request removal of this rejection.

§103 Rejection over Intelisano or Braswell in view of Facino and over Facino

Examiner also rejected claims 7, 8, 10 and 18-20 under 35 U.S.C. §103(a) over Intelisano or Braswell in view of Facino and over Facino. Specifically, Examiner maintained that the foregoing references as a whole teach a method of inducing phase II enzymes in subjects comprising administering a chloroform soluble *Echinacea purpurea* extract. These references were previously used on obviousness grounds in the Final Office Action of April 18, 2003. Applicants appealed that Action and filed an Appeal Brief on September 18, 2003 asserting that these references alone or in combination make Applicants' invention obvious. Before the Appeal was decided, Examiner re-opened prosecution by issuing a §112 rejection. Applicants, again, respectfully traverse the §103 rejections as conceivably applied to the amended claims.

Braswell states that "certain *Echinacea* extracts have shown direct anti-cancer activity *in vivo*" (column 2, lines 61-64). Facino discloses a method of purifying *Echinacea angustifolia* to obtain a chloroform fraction that has anti-hyaluronidase activity at a certain concentration and acidity (p.1450-1452). Intelisano discloses a food supplement that contains *Echinacea* for an antioxidant and suggests that *Echinacea angustifolia* and *Echinacea purpurea* are interchangeable.

A. A *Prima Facie* Case Of Obviousness Has Not Been Properly Established.

Applicants respectfully submit that Examiner has not established a proper *prima facie* case of obviousness because Braswell or Intelisano is not properly combinable with Facino as there is no reasonable expectation of success in modifying Facino's or substituting Facino's *Echinacea angustifolia* for Braswell or Intelisano's *Echinacea*. Further, this combination does not teach or suggest all the claim limitations, namely,

inducing phase II enzymes with chloroform-soluble *Echinacea purpurea* fractions. The concentration of actives responsible for *Echinacea*'s beneficial effects vary from species to species. It is known by one of ordinary skill in the art that a chloroform fraction from any *Echinacea* species, at any acidity, and at any concentration would not necessarily achieve the same levels of phase II activity and anti-hyaluronidase activity as suggested by Examiner. In fact, the data in support of anti-hyaluronidase activity in Facino and the data in support of Applicant's phase II activity demonstrate that different results can be achieved with different materials and concentrations. See arguments in Section B herein. Because several variables can alter the ability of *Echinacea* to induce phase II enzymes, there is no reasonable expectation of success. The modification or combination of the prior art make it, at best, obvious to try the claimed method but do not make the claimed method for inducing phase II with chloroform soluble fractions of *Echinacea purpurea* obvious without hindsight reconstruction. Obvious to try is not a sufficient basis for a *prima facie* case.

Additionally, a *prima facie* case has not been properly established because all the claimed limitations are not met. See MPEP §2143.03. None of the cited references teach or suggest the specific method of inducing phase II by administering chloroform-soluble *Echinacea purpurea* fractions selected from either chloroform root fractions or acidic chloroform aerial fractions. Because none of the cited references teach or suggest the claimed method for inducing phase II enzymes, the rejection under §103(a) is improper and this rejection should be withdrawn.

B. Applicant's Method Provides Surprising Results.

Even if the references are properly combined, Applicants believe that the claimed invention is not obvious over the cited art because Applicants' invention provides surprising results as outlined throughout the present specification. MPEP §2144.09 states that, "A *prima facie* case of obviousness based on structural similarity is rebuttable by proof that the claimed compounds possess unexpectedly advantageous or superior properties." Applicants have found that the chloroform-soluble chloroform portion of *Echinacea purpurea* performs surprisingly better than other *Echinacea purpurea* fractions in inducing phase II activity. The phase II inducing activity of the chloroform-soluble fraction is greater than one would expect given the prior art teachings. A greater

than expected result is an evidentiary factor pertinent to the legal conclusion of obviousness...of the claims at issue. See MPEP §716.02(a) and In re Corkill, 711 F.2d 1496, 226 USPQ 1005 (Fed. Cir. 1985). In the present case, the chloroform fraction (at a neutral pH) of Facino's *angustifolia* shows inferior hyaluronidase inhibition when compared with the ethylacetate fraction (page 1451, paragraph 4 and page 1452, Fig. 1). As such, if one assumed that *purpurea* and *angustifolia* were interchangeable and affected hyaluronidase inhibition and phase II enzyme induction equally (as suggested by Examiner), one would expect that the chloroform root fraction of Applicants' *Echinacea purpurea* would be inferior to the ethylacetate fraction. In contrast, Applicants' chloroform root fraction provides surprisingly superior phase II induction results than the ethylacetate fraction as shown in Fig. 2 of the present application. Moreover, Applicants' claimed method provides surprising results because the level of enzyme activity in the chloroform root fraction was 35% higher than the root methanol fraction; the acidic chloroform aerial fraction was 87% higher than the more polar methanol fraction (see p.5, lines 15-20). At 0.09mg/ml of *Echinacea purpurea* extract, the chloroform root fraction had 1.86 times the quinone reductase activity of the untreated control (page 9, lines 26-28). Therefore, Applicants believe that the claimed method of inducing phase II enzyme by administering a chloroform root fraction or an acidic chloroform aerial fraction of *Echinacea purpurea* is not obvious and is patentable over the prior art.

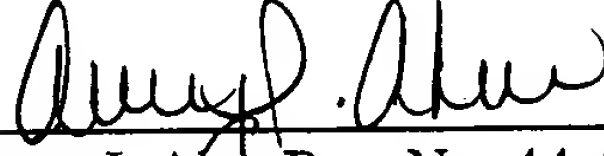
Conclusion

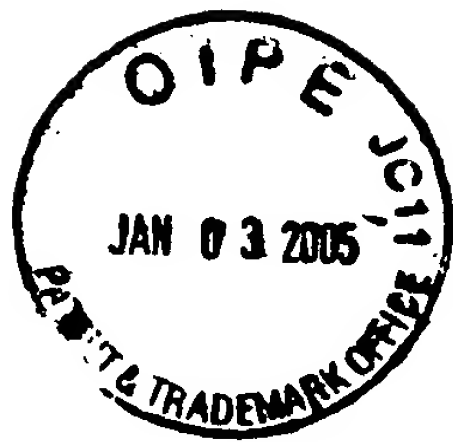
Applicants have made an earnest effort to place their application in proper form and to distinguish their invention as presently claimed from the cited prior art. Therefore, Applicants respectfully request entry of the amendments and allowance of the pending claims. It is also respectfully requested that the Examiner expeditiously notify the undersigned attorney as to the disposition of the arguments presented herein in accordance with MPEP §714.13.

Respectfully submitted,

By: Brovelli et al.

10/15/04
Date


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Patent
NP4053

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Brovelli et al.
Serial No. : 09/575,307
Filing Date : May 19, 2000
Examiner : Michael V. Meller
Group Art : 1654
For : ECHINACEA INDUCTION OF PHASE II ENZYMES

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

I, ERNESTO BROVELLI, declare the following:

1. I am a co-inventor of the above-identified patent application.
2. I received a B.S. in Agronomy from National University of Rosario, Argentina in 1983. I received a M.S. in Horticulture from the University of Nebraska, Lincoln in 1991. I received a Ph. D. in Horticulture from the University of Florida in 1997.
3. I have been employed with Access Business Group LLC, an affiliated corporation of Altacor Inc. (formerly Amway Corporation) since September 15, 1997 as a Research Scientist; promoted in 2000 to Senior Scientist and in 2002 to Group Leader. Prior to being employed with Access Business Group LLC, I was employed with the University of Florida as a Research Consultant and as a Department Director at the Secretary of Agriculture in Buenos Aires, Argentina.
4. I have authored or co-authored scientific publications relating to plant based compounds. Some of my publications are listed in Exhibit 1.

5. I have read the office action mailed June 30, 2004 issued in connection with the subject application. I have been told that Claims 7, 8, 10, and 18-20 have been rejected under §112, paragraph 1, for failing to comply with the enablement requirement because the quinone reductase ("QR") *in vitro* assay used in the present invention is not predictive of *in vivo* phase II activity.
6. I am familiar with the QR assay used in the present invention, which has been used by myself and others under my supervision, to test phase II activity of plant based compounds.
7. The QR assay is a well known and a widely used bioassay of phase II activity, the basic protocols of which are described in Prochaska, H.J. and Santamaria, A.B., Direct Measurement of NAD(P)H: Quinone Reductase from Cells Cultured in Microtiter Wells: A Screening Assay for Anticarcinogenic Enzyme Inducers, *Analytical Biochemistry*, 169:328-336 (1988).
8. The QR assay in Prochaska, H.J. was designed to predict *in vivo* phase II activity. See Prochaska, H.J., *supra* at 329. The QR assay used in the present invention was modeled after Prochaska, H.J.
9. Phase II activity identified by QR assays of plant based compounds are known to be predictive of phase II activity *in vivo*. For example, sulforaphane from broccoli has been shown to exhibit phase II activity in a QR assay using murine hepatoma cells *in vitro*. In the same study, sulforaphane was administered to female mice to confirm that phase II induction occurred *in vivo*. Zhang, Y., Talalay, P., Cho, C., and Posner, G. H., A Major Inducer of Anticarcinogenic Protective Enzymes from Broccoli: Isolation and Elucidation of Structure, *Proc. Natl. Acad. Sci. USA*, 89:2399-2403 (1992).
10. Other publications supporting *in vitro* results correlating with *in vivo* results that were available prior to the filing date of the present invention include: (a) Prochaska, H.J.,

Santamaria, A.B., and Talaly, P., Rapid Detection of Inducers of Enzymes that Protect Against Carcinogens, *Proc. Natl. Acad. Sci. USA*, 89:2394-2398 (1992); and (b) Morel, F., Langouet, S., Maheo, K., and Guillouza, A., The Use of Primary Hepatocyte Cultures for the Evaluation of Chemoprotective Agents, *Cell Biology and Toxicology* 13:3232-329 (1997).

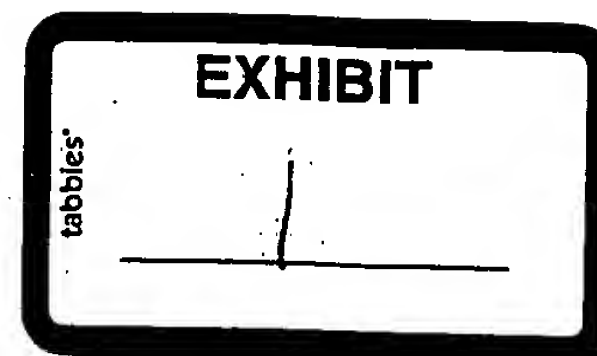
All publications cited herein form part of this Declaration (Exhibit 2).

11. Thus, it is my considered scientific opinion that chloroform-soluble *Echinacea purpurea*, shown to have phase II activity *in vitro*, is expected to have phase II activity *in vivo*.
12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application.

Dated: 10-3-04



Dr. Ernesto Brovelli



DECLARATION UNDER 37 C.F.R. §1.132

EXHIBIT 1

PUBLICATIONS

Non-Refereed

Leguizamón, E.S., E.A. Brovelli, E. Giuggia, and L. Allieri. 1985. Dinámica poblacional de Sorgo de Alepo (*Sorghum halepense*) en la secuencia trigo/soja. Evaluación del banco de propágulos. Impacto del control químico. Diagrama de caída de semillas. IDIA 438:44-48.

Brovelli, E.A. and H. Alvarez. 1988. Nemátodes en horticultura: un problema creciente. La Capital Rural, 5-22.

Brovelli, E.A. and R. Howell. 1991. Management of Food-transmitted Infections and Intoxications. J. Dairy Food and Environ. Sanit. 11:302-304.

Brovelli, E.A. 1992. Envasado de frutas y hortalizas. Clarín Rural, 6-27.

Hodges, L., R. Uhlinger., E. Brovelli, S. Cuppett and A. Parkhurst. 1992. Performance of Green and Blanched Asparagus Cultivars in Nebraska. HortScience 27:134 (abstract).

Brovelli, E.A. y G. Cohen. 1992. Análisis de la investigación hortícola nacional según los trabajos presentados en la AS.A.HO. Resúmenes del XV Congreso Argentino de Horticultura.

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Brovelli, E.A., J.K. Brecht, W.B. Sherman and C.A. Sims. 1995. Quality Profile of Fresh Market Melting and Nonmelting Peach Fruit. 1995 Proc. Fla. State Hort. Soc. 108:309-311.

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Brovelli, E.A., J.K. Brecht, W.B. Sherman and C.A. Sims. 1996. Sensory Principal Components and Analytical Aspects of Melting and Nonmelting-Flesh Peaches for the Fresh Market. The Institute of Food Technologists Book of Abstracts: 90-91 (abstract).

Brovelli, E.A., J.K. Brecht, W.B. Sherman and C.A. Sims. 1997. Response of Melting- and Nonmelting-Flesh Peach Genotypes to Postharvest Chilling. The Institute of Food Technologists Book of Abstracts: 29 (abstract).

Brovelli, E.A., J.L. Supri and P. Bubrick. 1998. Differences in Color and Flavonoid Content between Stems and Leaves of Passion Flower Plants. The Institute of Food Technologists Book of Abstracts: 77 (abstract).

Brovelli, E.A., J.K. Brecht, W.B. Sherman and C.A. Sims. 1998. A Contrast in Ethylene Production and Respiration between Melting -and Nonmelting-Flesh Peaches. HortScience 33:468 (abstract).

Brovelli, E.A., J. Rana, K. Chui and G. Menon. 1999. Image Analysis and Phytochemical Profile of *Echinacea purpurea* Herb as Affected by Drying Conditions. The Institute of Food Technologists Book of Abstracts: 56 (abstract).

Omary, M.B., P. David, T. Kizer, E. Brovelli, J.C. Araya, D. Pusateri, and R. Rutledge. 1999. Sulforaphane and Vitamin C Concentration in Developing Florets and Leaves of Broccoli. 1999 American Chemical Society Book of Abstracts (abstract).

Brovelli, E.A. and R.M. Johnson. 2000. Postharvest Alterations of *Echinacea purpurea* herb. Postharvest 2000 Book of Abstracts (abstract).

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Gellenbeck, K., K. Randolph, K. Stonebrook, E. Brovelli, Y. Qian, D. Bankaitis-Davis. 2002. Human *In Vitro* and *In Vivo* Immune Gene Expression as Influenced by a Commercial Blended *Echinacea* Product. 2002 FASEB Experimental Biology Meeting, New Orleans, Louisiana (abstract).

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Brovelli, E.A. 2004. Different Paths for the Fresh-Market and the Nutraceutical Industry. Fifth International Postharvest Symposium Volume of Abstracts:40 (abstract).

Refereed

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Omary, M.B., Brovelli, E.A., Pusateri, D.J, David, P., Rushing J.W. and J.M. Fonseca. 2003. Sulforaphane Potential and Vitamin C Concentration in Developing Heads and Leaves of Broccoli (*Brassica oleracea* var. *italica*). J. Food Qual. 26:523-530.

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Randolph, R.K., Gellenbeck, K., Stonebrook, Brovelli, E., Qian, Y., Bankaitis-Davis, D. and J. Cheronis. 2003. Regulation of Human Immune Gene Expression as Influenced by a Commercial Blended *Echinacea* Product: Preliminary Studies. *Exp. Biol. And Med.* 228:1051-1056.

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Omary M.B., Krueger, R.R., Y. Li, E.A. Brovelli, and H.F. Florez. 2004. Bioflavonoid Profile of Exotic Citrus Species. *Acta Hort.* 629:449-455.

Direct Measurement of NAD(P)H:Quinone Reductase from Cells Cultured in Microtiter Wells: A Screening Assay for Anticarcinogenic Enzyme Inducers

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Received August 27, 1987

We describe a rapid and direct assay of NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) activity in cultured cells suitable for identifying and purifying inducers of this detoxication enzyme. Hepa 1c1c7 murine hepatoma cells are plated in 96-well microtiter plates, grown for 24 h, and exposed to inducing agents for another 24 h. The cells are then lysed and quinone reductase activity is assayed by the addition of a reaction mixture containing an NADPH-generating system, menadione (2-methyl-1,4-naphthoquinone), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Quinone reductase catalyzes the reduction of menadione to menadiol by NADPH, and MTT is reduced nonenzymatically by menadiol resulting in the formation of a blue color which can be quantitated on a microtiter plate absorbance reader. The reaction is more than 90% dicoumarol inhibitable and menadione dependent. The results are comparable to those obtained by harvesting cells from larger plates, preparing cytosols, and carrying out spectrophotometric measurements. © 1988 Academic Press, Inc.

KEY WORDS: quinone reductase; phase II enzymes; enzyme induction; microtiter plates; anticarcinogens.

We have developed a rapid, efficient, and inexpensive assay for measuring NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) from cells cultured in microtiter wells. Quinone reductase² is a widely distributed, primarily cytosolic, dicoumarol-inhibitable flavoprotein that catalyzes the reduction of a wide variety of quinones and quinoneimines (1,2). Quinone reductase protects cells against the toxicity of quinones and their metabolic precursors by promoting the obligatory two-electron reduction of quinones to hydroquinones which are then sus-

ceptible to glucuronidation (3-10). In addition, quinone reductase is induced coordinately with other electrophile-processing Phase II enzymes (glutathione *S*-transferases and UDP-glucuronosyltransferases) by a variety of compounds that protect rodents from the toxic, mutagenic, and neoplastic effects of carcinogens (2,11-13). There is a large body of evidence which suggests that the induction of Phase II enzymes is the predominant mechanism by which these heterogeneous compounds are chemoprotective (11-15), and it is clear that the monitoring of Phase II enzyme induction is a convenient method for screening for anticarcinogenic activity (11-13,15-23).

Although many anticarcinogenic enzyme inducers have been discovered, other unrecognized compounds may exist that are potent, effective, and nontoxic (e.g., the active constituents from poorly characterized plant

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EXPERIMENTAL

Materials

MTT, NADH, serum albumin, phosphate, bakers' hydrogenase, tomycin, and from Sigma. NADH was from Piscataway (Piscataway, NJ) and fetal calf serum (Grand Island, NY). Nitrile were (Muskegon, MI). benzo-*p*-diox (IIT Research

¹ To whom reprint requests should be addressed.

² Abbreviations used: quinone reductase, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2); DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sudan I, 1-phenylazo-2-naphthol; Sudan II, 1-(2,4-dimethylphenylazo)-2-naphthol; Sudan III, 1-(4-phenylazophenylazo)-2-naphthol.

extracts) (11,17,20-23). Unfortunately, screening compounds for their ability to induce Phase II enzymes in animals is difficult, time-consuming, and expensive (17,20). Our laboratory has recently developed a more rapid screening system by demonstrating that quinone reductase is induced in the Hepa 1c1c7 murine hepatoma cell line by many of the compounds that induce Phase II enzymes *in vivo* (24-26). Nevertheless, conventional assay techniques (e.g., harvesting, homogenizing, centrifuging, and assaying for enzymatic activity and protein content) are time-consuming and therefore limit the usefulness of this procedure. For this reason, we have developed a direct assay of quinone reductase from cells grown and induced in 96-well microtiter plates by measuring the NADPH-dependent menadiol-mediated reduction of MTT. This assay procedure is rapid, accurate, inexpensive, capable of screening many compounds and/or a series of concentrations of compounds in a single experiment, and amenable to computerized data processing. This method should facilitate the identification of new and potentially important chemoprotective compounds of medicinal interest.

EXPERIMENTAL PROCEDURES

Materials

MTT, NADP, FAD, menadione, bovine serum albumin, Tris base, glucose 6-phosphate, bakers' yeast glucose-6-phosphate dehydrogenase, Tween-20, penicillin G, streptomycin, and crystal violet were obtained from Sigma Chemical Co. (St. Louis, MO); NADH was from Pharmacia P-L Biochemicals (Piscataway, NJ); microtiter wells were from Falcon (Becton-Dickinson Labware, Oxnard, CA); α -minimal essential medium and fetal calf serum were from GIBCO (Grand Island, NY); and DMSO and acetonitrile were from Burdick and Jackson (Muskegon, MI). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from IIT Research Institute (Chicago, IL). Other

inducing agents were obtained and prepared as described previously (24-26). Hepa 1c1c7 cells were a gift of J. P. Whitlock, Jr., Stanford University. Multiple pipettors (50- and 200- μ l Octapipets) were purchased from Costar (Cambridge, MA). The absorbances of microtiter wells were measured with an automated optical scanner equipped with a 610-nm filter (Biotek, Winooski, VT).

Methods

Growth of cells. Hepa 1c1c7 cells were plated at a density of 10 000 cells/well in 200 μ l of α -minimal essential medium (without ribonucleosides or deoxyribonucleosides) supplemented with 10% fetal calf serum. The cells were grown for 24 h in a humidified incubator in 5% CO₂ at 37°C. The medium was decanted and each well was refed with 200 μ l of α -minimal essential medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, 100 μ g/ml of streptomycin, and 0.1% DMSO. Compounds to be tested as inducers were dissolved in DMSO and were diluted into the media so that the final concentration of DMSO was 0.1% by volume. Control cells were always grown in the second column of wells and were fed media containing 0.1% DMSO. The cells were then incubated for an additional 24 h.

Assay of quinone reductase. The following stock solution was prepared for each set of assays: 7.5 ml of 0.5 M Tris-Cl (pH 7.4), 100 mg of bovine serum albumin, 1 ml of 1.5% Tween-20, 0.1 ml of 7.5 mM FAD, 1 ml of 150 mM glucose 6-phosphate, 90 μ l of 50 mM NADP, 300 U of yeast glucose-6-phosphate dehydrogenase, 45 mg of MTT, and distilled water to a final volume of 150 ml. Menadione (1 μ l of 50 mM menadione dissolved in acetonitrile per milliliter of reaction mixture) was added just before the mixture was dispensed into the microtiter plates.

After the plates were exposed to test compounds for 24 h, the media were decanted, and the cells were lysed by incubation at 37°C for 10 min with 50 μ l in each well of a solution containing 0.8% digitonin and 2

mm EDTA, pH 7.8. The plates were then agitated on an orbital shaker (100 rpm) for an additional 10 min at 25°C, after which 200 μ l of the complete reaction mixture was added to each well with the aid of a multiple pipetting device (Octapipet). A blue color developed and the reaction was arrested after 5 min by the addition of 50 μ l of a solution containing 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate, pH 7.4. The plates were then scanned at 610 nm. The first column of wells in the plates always contained the reaction mixture only and served as the nonenzymatic blank. The average absorbance value of this column of wells was subtracted automatically from all other absorbance readings.

In order to determine the proportion of MTT reduction attributable to quinone reductase activity (Table 1), three sets of microtiter plates were grown and induced under identical conditions. The cells on one set of plates were assayed as described above. A second set of cell lysates was assayed in the presence of 50 μ l per well of 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4). The third set of cells was lysed in the standard fashion but assayed with a reaction mixture containing no men-

adione. The absorbances were scanned 5 min after the addition of the reaction mixture.

Crystal violet staining. Since some quinone reductase inducers or crude fractions that are being screened for inducer activity depress the rate of cell growth, it is desirable to relate the observed quinone reductase activity to the number of cells or the amount of protein in each microtiter well. This normalization can be conveniently accomplished by staining a set of microtiter plates treated identically to those used for the MTT assay with crystal violet. We have used a slight modification of the method of Drysdale *et al.* (27) for this purpose. The media were decanted, the plates were submerged in a vat of 0.2% crystal violet in 2% ethanol for 10 min and rinsed for 2 min with tap water, and the bound dye was solubilized by incubation at 37°C for 1 h with 200 μ l of 0.5% sodium dodecyl sulfate in 50% ethanol. The plates were then scanned at 610 nm.

In order to demonstrate the validity of the crystal violet assay as a convenient measure of protein content and cell number, five twofold serial dilutions of Hepa 1c1c7 cells were plated in each of six identical 24-well 2-cm² plates (four wells per dilution of cells). The cells were grown for 24 h, refed with

TABLE 1
RATES OF MTT REDUCTION OF CONTROL, β -NAPHTHOFILAVONE OR 1,2-DITHIOL-3-THIONE-TREATED
HEPA 1c1c7 CELLS GROWN IN MICROTITER WELLS

Treatment of cells	Number of wells assayed	Change in absorbance ($\times 10^3$) in 5 min at 610 nm		
		Standard assay	Standard assay with prior dicoumarol addition	Standard assay minus menadione
Control	16	212 \pm 12 ^a	16.1 \pm 4.4	22.0 \pm 2.8
β -Naphthoflavone (2 μ M)	8	862 \pm 20	17.3 \pm 4.0	17.3 \pm 4.0
1,2-Dithiol-3-thione (10 μ M)	8	453 \pm 20	20.3 \pm 3.4	20.3 \pm 3.4

Note. Hepa 1c1c7 cells were grown and induced in three parallel sets of microtiter wells as described under Materials and Methods. One set of plates was lysed and assayed in the standard fashion, another set was assayed in the presence of 50 μ l of 0.3 mM dicoumarol per well, and the third set was assayed with reaction mixture containing no menadione.

^a Mean values \pm standard deviations.

COMP GROWN IN

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 β -Naph
Benzo[
3-Meth

Azo dyes
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1-(2-Py
1-(2-Th
Sudan I
Sudan I
Sudan I

Diphenols
Catecho
Resorcin
Hydroq
tert-But
tert-But
tert-But

Isothiocya
Benzylis

Dithiolthio
1,2-Ditl
4-Pheny
5-(2-Py
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TABLE 2

COMPARISON OF QUINONE REDUCTASE INDUCTIONS OBTAINED BY DIRECT ASSAY OF HEPA 1c1c7 CELLS GROWN IN MICROTITER WELLS AND BY CONVENTIONAL ASSAY OF HEPA 1c1c7 CELLS GROWN ON 75-cm² PLATES

Compounds	Concentration (μM)	Ratio quinone reductase specific activity (treated/control)	
		Direct assay in microtiter wells ^a	Conventional assay with 75-cm ² plates ^b
Polycyclic aromatics			
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin	0.01	3.10 ± 0.51 ^c	2.95 ± 0.40 ^{c,d}
β-Naphthoflavone	2	4.66 ± 0.25	3.56 ± 0.34 ^d
Benzo[<i>a</i>]pyrene	2	3.80 ± 0.34	3.58 ± 0.08 ^d
3-Methylcholanthrene	2	3.43 ± 0.40	3.29 ± 0.24 ^d
Azo dyes			
1,1'-Azonaphthalene	2	5.08 ± 0.36	4.47 ± 0.52 ^d
1-(2-Pyridylazo)-2-naphthol	2	4.80 ± 1.07	3.61 ± 0.26 ^d
1-(2-Thiazolylazo)-2-naphthol	2	3.25 ± 0.34	3.00 ± 0.18 ^d
Sudan I	2	4.25 ± 0.31	3.36 ± 0.12 ^d
Sudan II	2	2.90 ± 0.20	2.54 ± 0.20 ^d
Sudan III	2	1.80 ± 0.08	2.28 ± 0.20 ^d
Diphenols			
Catechol	30	1.98 ± 0.11	1.79 ± 0.20 ^c
Resorcinol	30	1.09 ± 0.08	0.88 ± 0.04 ^c
Hydroquinone	30	2.35 ± 0.19	1.92 ± 0.12 ^c
<i>tert</i> -Butylcatechol	30	1.75 ± 0.17	1.65 ± 0.10 ^c
<i>tert</i> -Butylresorcinol	30	0.97 ± 0.08	0.79 ± 0.08 ^c
<i>tert</i> -Butylhydroquinone	30	2.66 ± 0.23	2.87 ± 0.38 ^c
Isothiocyanate			
Benzylisothiocyanate	5	1.91 ± 0.20	3.16 ± 0.62 ^d
Dithiolthiones			
1,2-Dithiol-3-thione	10	2.65 ± 0.25	2.95 ± 0.40 ^d
4-Phenyl-1,2-dithiol-3-thione	30	3.53 ± 0.34	3.46 ± 0.56 ^d
5-(2-Pyrazinyl)-4-methyl-1,2-dithiol-3-thione	30	1.59 ± 0.14	2.32 ± 0.34 ^d

^a Direct assay described under Materials and Methods (*N* = 8).

^b Hepa 1c1c7 cells grown, treated, and assayed from 75-cm² plates as described by DeLong *et al.* (25).

^c Mean value ± standard deviation.

^d Unpublished results (*N* = 4).

^e From Prochaska *et al.* (24).

medium, and grown for an additional 24 h. The total cellular protein of each well of one plate was determined. The wells in this plate were washed with phosphate-buffered saline, 400 μl of water was added, and the wells were sonicated. Aliquots from each well were assayed by the method of Bradford (28), with bovine serum albumin as standard. A second plate was used to determine cell number per

well, and the remaining four plates were stained with crystal violet and destained as described for the 96-well plates. The stain from each well was solubilized in 3 ml of 0.5% sodium dodecyl sulfate in 50% ethanol and the absorbance of the resulting solution was measured in 1.0-cm light path cuvettes at 610 nm. The average absorbance for every concentration of cells from each individual

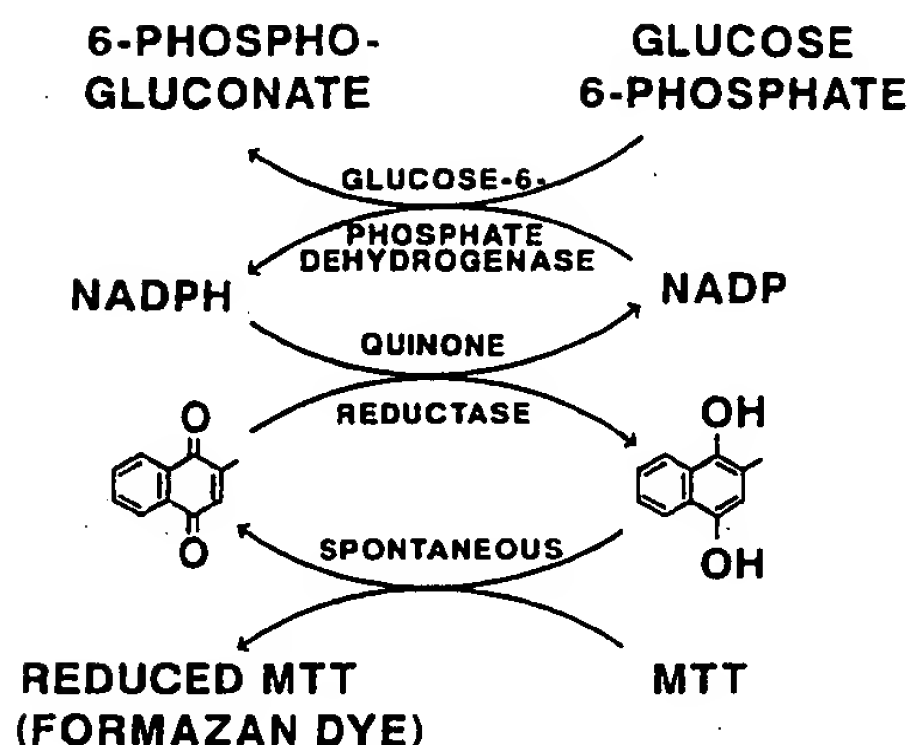


FIG. 1. Principle of the assay of quinone reductase. Glucose 6-phosphate and glucose-6-phosphate dehydrogenase continually generate NADPH, which is used by quinone reductase to transfer electrons to menadione. The menadiol reduces MTT to the blue formazan which can be measured over a broad range of wavelengths (550–640 nm). The complete reaction mixture is described under Materials and Methods. Both NADPH and menadione are regenerated, which obviates problems encountered with substrate depletion.

plate was used to determine the mean and standard error of absorbances shown in Fig. 4.

Determination of specific activities. The results of quinone reductase specific activity measurements for Table 2 are reported as the ratios of specific activities of inducer-treated microtiter wells to those of controls. The rate of MTT reduction and the crystal violet absorbances for the inducer-treated groups were compared to those of control cells grown on the same microtiter plates. The results were calculated using a spread-sheet program and the standard deviations shown in Table 2 were determined from the standard deviations of both the MTT and crystal violet assays.

RESULTS AND DISCUSSION

The assay (Fig. 1) is based on the production of a blue color when MTT is reduced nonenzymatically by menadiol that is generated enzymatically from menadione by quinone reductase. Similar systems have been

used for staining quinone reductase activity in gels (29). Although the bleaching of the color of 2,6-dichloroindophenol (a substrate for quinone reductase) by reduced nicotinamide nucleotides can be followed in microtiter wells, its use is unsatisfactory in this assay system for two main reasons. First, the depletion of 2,6-dichloroindophenol results in the significant decline of reaction rate with time. Second, small errors in pipetting of the reaction mixture containing 2,6-dichloroindophenol (which has an absorbance of 1.8 to 2.0 under usual assay conditions) can result in significant variability. These errors adversely affect the reproducibility of data since only the *absolute* absorbance at 5 min rather than the absorbance *change* in each well can be conveniently measured. The use of MTT reduction avoids these difficulties since (a) the menadione concentration remains constant in the assay system because MTT reduction results in menadione regeneration and (b) the assay depends on the generation of color from absorbances that are initially negligible. Thus, all wells have negligible absorbance at zero time and the absolute ab-

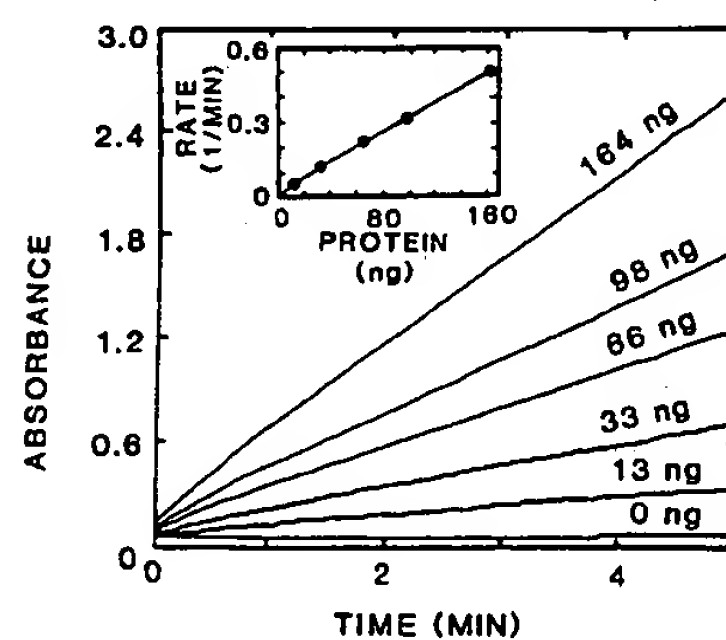


FIG. 2. Dependence of MTT reduction rate on amount of quinone reductase. Pure murine quinone reductase (30) was added in the indicated quantities and the rates of MTT reduction were recorded for 5 min at 610 nm in 1.0-cm light path cuvettes and in total volumes of 3.0 ml. The reaction mixture was identical to that used for microtiter well assays. Menadione was added to initiate the reaction. The assay was linear for 5 min for absorbance changes of up to 0.5/min. The rates obtained were proportional to the amount of enzyme added (inset; $r = 0.999$).

sorbance at change in ab extinction co found to be

The assay system that concentration sired. Because the number grown in a measure concentration is chosen ($\approx \frac{1}{2}$) for this assay promoted by genases that able nor me use of NAD rates. With dione-indep tive reduction less than 10% the standard of reduction diaphorases) presence of c menadione, of the specific Because the by NADPH parallel dic routine screen

Since both absorbance amount of a sorbance change rate of absorption enzyme is 0 of MTT reduction microtiter well of NADH standard method chaska and of dicoumar the cuvette used for the tually instantaneous reduction. Th

sorbance at 5 min accurately reflects the change in absorbance during this time. The extinction coefficient of reduced MTT was found to be $11,300 \text{ M}^{-1} \text{ cm}^{-1}$ at 610 nm.

The assay utilizes an NADPH generating system that maintains a constant NADPH concentration, which can be varied as desired. Because the rate of MTT reduction by the number of Hepa 1c1c7 cells normally grown in a microtiter well is too rapid to measure conveniently if the NADPH concentration is saturating, $24 \mu\text{M}$ NADPH was chosen ($\approx \frac{1}{2} K_m$; (30)). NADH is unsuitable for this assay because MTT reduction is also promoted by other NADH-linked dehydrogenases that are neither dicoumarol inhibitable nor menadione dependent. Thus, the use of NADH results in high nonspecific rates. With NADPH, however, the menadione-independent or dicoumarol-insensitive reduction of MTT with control cells was less than 10% of the total activity obtained in the standard assay (Table 1). This slow rate of reduction of MTT (caused by nonspecific diaphorases) is almost the same in both the presence of dicoumarol and in the absence of menadione, and it is unaffected by induction of the specific NAD(P)H:quinone reductase. Because the basal rate of reduction of MTT by NADPH is low, there is no need to run parallel dicoumarol-inhibited plates during routine screening.

Since both substrates are regenerated, the absorbance is linearly proportional to the amount of added enzyme up to a rate of absorbance change of 0.5 per min (Fig. 2). The rate of absorbance change in the absence of enzyme is 0.001/min. Furthermore, the rate of MTT reduction in the assay utilized for microtiter wells is linearly related to the rate of NADH oxidation as measured in the standard menadione assay described by Prochaska and Talalay (30; Fig. 3). The addition of dicoumarol ($50 \mu\text{M}$ final concentration) to the cuvette under conditions similar to those used for the microtiter assay results in virtually instantaneous inhibition of MTT reduction. The rapidity of inhibition is to be

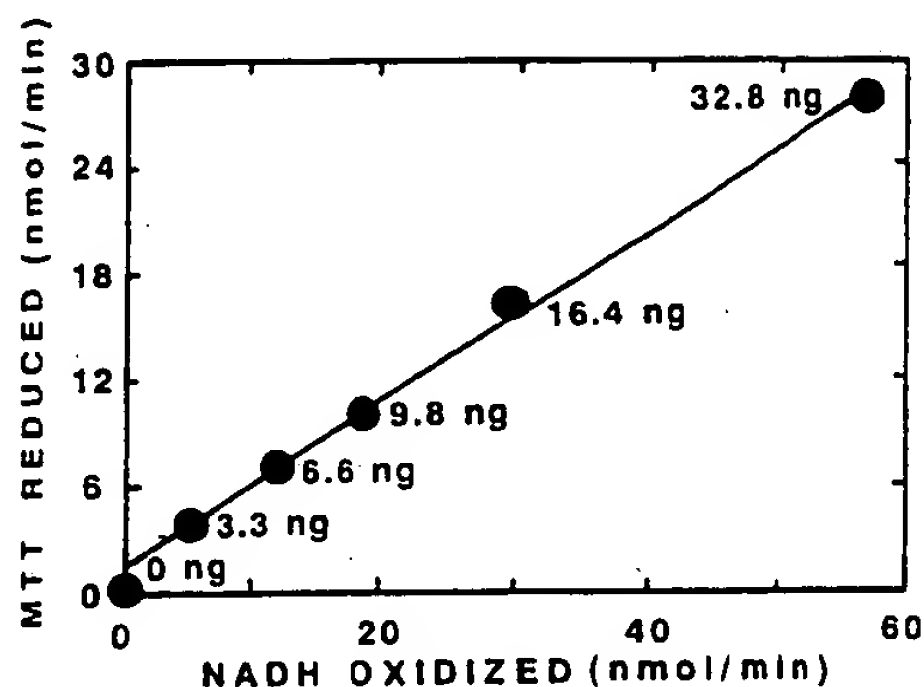


FIG. 3. Comparison of quinone reductase activity measured by the NADPH-dependent menadiol-mediated reduction of MTT with the standard quinone reductase assay. Six amounts of pure quinone reductase (30) indicated in the figure were assayed in the standard assay for quinone reductase as described by Prochaska and Talalay (30) by following the oxidation of NADH ($200 \mu\text{M}$) by menadione ($50 \mu\text{M}$) at 340 nm, as well as in the assay system utilized for microtiter wells by following the reduction of MTT by NADPH at 610 nm. Both assays were performed at 25°C in 1.0-cm light path cuvettes and in total volumes of 3.0 ml. The rates obtained by the two assays are linearly correlated ($r = 0.998$) and the MTT assay rate was 46.7% that of the standard NADH assay.

expected since the K_i value for dicoumarol is low (110 nM) and the concentration of the competing substrate (NADPH) is also low ($20 \mu\text{M}$) (30). Thus, this system provides an appropriate assay for quinone reductase.

Because we were interested in maximizing the rapidity with which the screening of inducers could be performed, we modified the method of crystal violet staining described by Drysdale *et al.* (27). This procedure has been used with great success to determine the specific activity of cytotoxic factors in the L929 murine fibroblast line, since it is a rapid, simple, and reliable method for determining cell number (27). Staining with crystal violet also appears to be well suited for the Hepa 1c1c7 murine hepatoma cell line since the degree of crystal violet absorption correlates well with cell number and total protein (Fig. 4; $r = 0.996$ and 0.997 , respectively). Indeed, at exceedingly high cell densities/

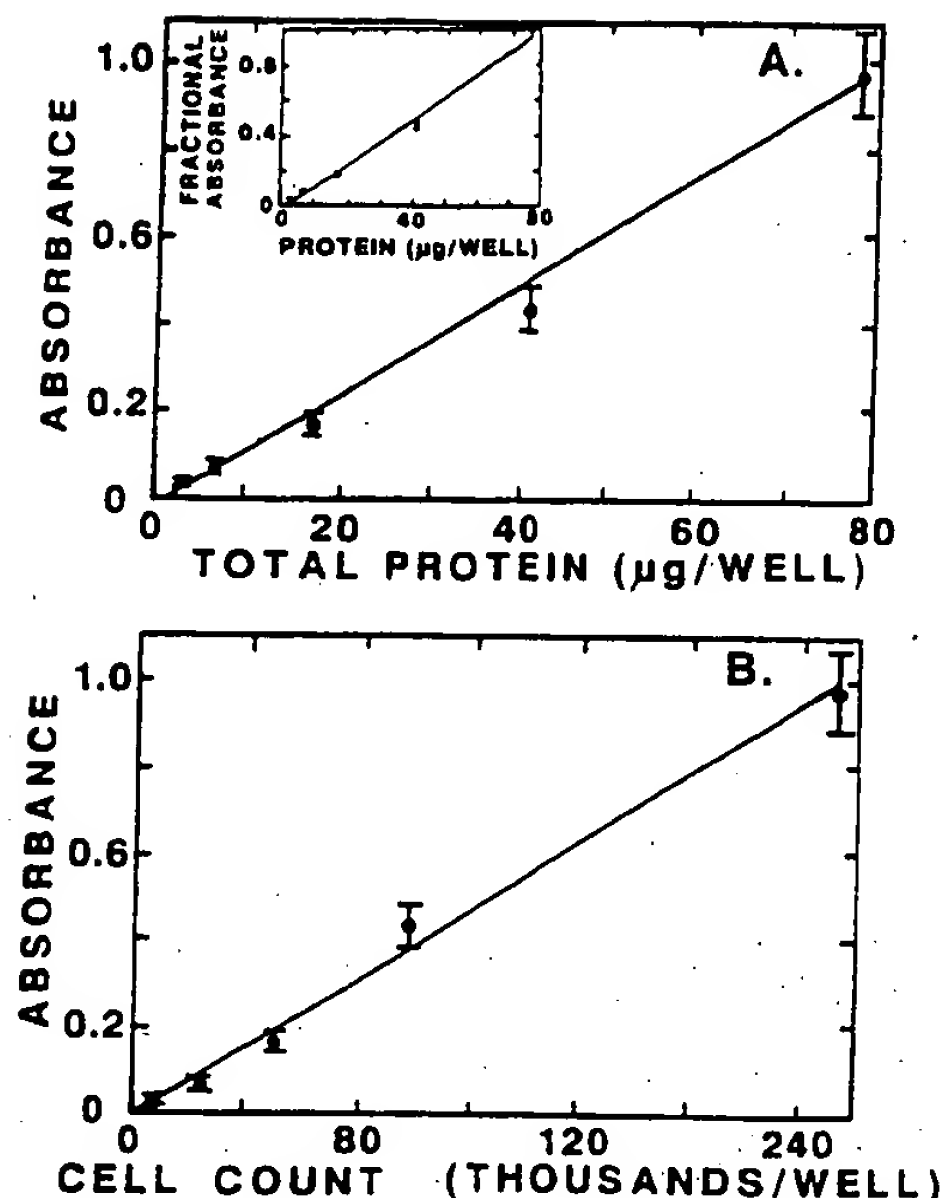


FIG. 4. Crystal violet staining correlates with total cellular protein (A) and cell number (B). Hepa 1c1c7 cells were plated at five cell densities in six identical 24-well 2-cm² plates and grown as described under Materials and Methods. Aliquots from one plate were used to estimate cell protein (presented as μg of protein per well) by the method of Bradford (28), and a second plate was used to determine cell number. The remaining four plates were stained, destained, and solubilized in 3 ml of 0.5% sodium dodecyl sulfate in 50% ethanol. The solutions were transferred to 1.0-cm light path cuvettes and the absorbances were then determined at 610 nm. The average crystal violet absorbance for every concentration of cells from each plate was determined, from which the mean absorbances and standard errors shown in the figure were calculated ($N = 4$). The absorbances of crystal violet were linearly correlated with total protein and cell number ($r = 0.997$ and 0.996 , respectively). Furthermore, the proportion of crystal violet absorbance relative to the highest absorbance was virtually the same between plates (inset).

cm², the crystal violet absorbance continues to correlate well with total protein, although the ratio of crystal violet absorbances to cell number increases (data not shown). Although there is some variability of the absolute absorbance of crystal violet between individual plates, the relative proportion of

staining of cells of different cell densities is remarkably constant (Fig. 4, inset). Hence, we find that crystal violet staining is a suitable method for the rapid estimation of total cellular protein and/or cell number and the data from Fig. 4 can be used to derive a simple formula for estimating quinone reductase specific activity.³ We found that over many ($N = 32$) experiments the specific activities ranged between 104 ± 3.4 and 355 ± 18.7 nmol/min/mg, and the mean \pm standard deviation of the averages is 208 ± 66 nmol/min/mg. The specific activity of quinone reductase in Hepa 1c1c7 cells grown in 75-cm² plates and assayed in the conventional manner with menadione (50 μM) and NADH (200 μM) as substrates ranged from 213 ± 6.6 to 578 ± 81.6 nmol/min/mg of protein. The mean and standard deviation of the averages

³ The specific activity of quinone reductase (nmol/min/mg of protein) can be estimated by using the extinction coefficient of MTT ($11,300 \text{ M}^{-1} \text{ cm}^{-1}$ at 610 nm) and the measure of total cellular protein as determined by the proportionality constant calculated from the calibration curve for crystal violet. This constant (37.8 ml/mg/cm light path at 610 nm) is the slope of the line depicted in Fig. 4A multiplied by 3.0 ml (the volume in which the crystal violet stain was solubilized). Because of the orientation of the light beam relative to the microtiter well, the absorbance of a given quantity of chromophore is independent of volume; i.e., the product of the concentration and path length is a constant. In other words, for a given amount of chromophore, if the concentration is halved by the addition of solvent, the path length is doubled and the total absorbance remains unchanged. Thus, the moles of MTT reduced or the mass of protein per well can be determined from their respective absorbances, the extinction coefficient or proportionality constant, and the area of the microtiter well (0.32 cm²). Furthermore, since both the MTT and crystal violet assays are scanned in microtiter wells of the same diameter, the specific activity calculation becomes independent of area. Therefore, specific activity can be calculated from the simple formula

specific activity

$$= \frac{\text{absorbance change of MTT/min}}{\text{absorbance of crystal violet}} \times 3345 \text{ nmol/mg.}$$

where 3345 nmol/mg is the ratio of the proportionality constant determined for crystal violet and the extinction coefficient of MTT.

BLANK
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FIG. 5. Ph
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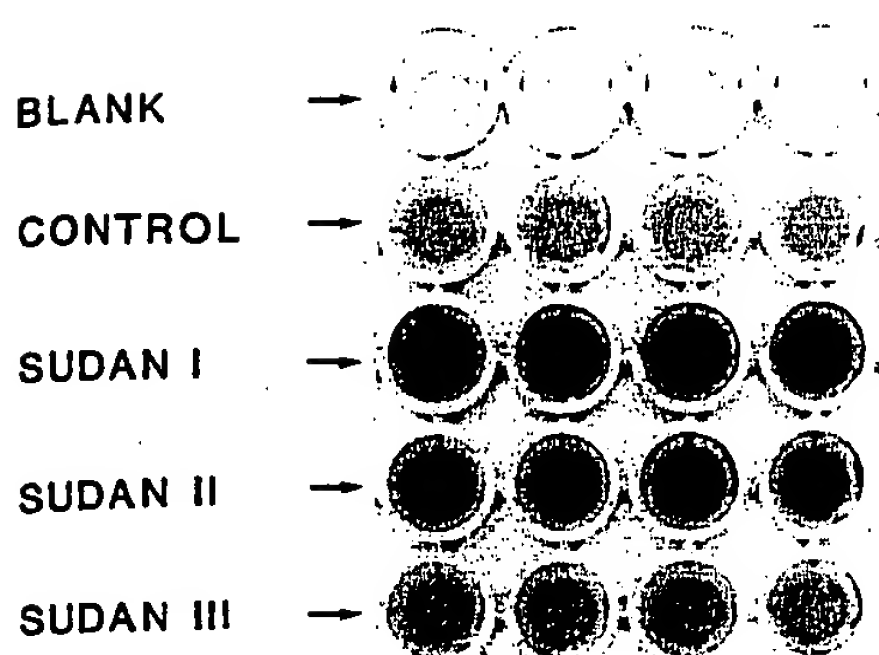


FIG. 5. Photograph showing the color (blue) that develops in the assays for quinone reductase activity of Hepa 1c1c7 cells grown in microtiter wells. The cells were grown, induced, and assayed as described under Materials and Methods. Blank wells contain no cells; control wells contain Hepa 1c1c7 cells treated with medium containing 0.1% DMSO (without inducer). Sudan I, II, and III wells contain cells that were treated with media containing the respective azo dye (2 μ M) in 0.1% DMSO. All cells were grown for 24 h and then treated with inducer or DMSO for another 24 h prior to assay. Four identical wells are shown for each condition.

of 15 experiments is 357 ± 106 nmol/min/mg of protein.

The usefulness of the microtiter system in screening for inducers of quinone reductase is illustrated in Table 2 and Fig. 5. This assay accurately identified inducers and noninducers and yielded virtually the same rank order of induction as did experiments with cells grown on 75-cm² plates and assayed in the conventional manner. For example, we have reported that resorcinol and its substituted congeners were inactive as inducers of quinone reductase, whereas catechols and hydroquinones could significantly elevate levels of quinone reductase in the Hepa 1c1c7 cell line (24). The same patterns were observed with the diphenols tested in the direct assay system (Table 2). Furthermore, the rank order of induction potency of azo dyes tested in the direct assay is the same as in the conventional assay system. Figure 5 demonstrates that the degree of quinone reductase induction can be detected without the assis-

tance of a microtiter scanner. Scanning of the absorbances for the experiment shown in Table 2 required less time than did harvesting of cells from the equivalent number of 75-cm² plates. Data processing can be further simplified by linking the scanner to a personal computer. We conclude that the direct assay of quinone reductase from cells grown in microtiter wells may facilitate the identification and isolation of novel inducers of chemoprotective enzymes such as quinone reductase.

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A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure

(chemoprotection/enzyme induction/isothiocyanates/sulforaphane/quinone reductase)

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ABSTRACT Consumption of vegetables, especially crucifers, reduces the risk of developing cancer. Although the mechanisms of this protection are unclear, feeding of vegetables induces enzymes of xenobiotic metabolism and thereby accelerates the metabolic disposal of xenobiotics. Induction of phase II detoxication enzymes, such as quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione *S*-transferases (EC 2.5.1.18) in rodent tissues affords protection against carcinogens and other toxic electrophiles. To determine whether enzyme induction is responsible for the protective properties of vegetables in humans requires isolation of enzyme inducers from these sources. By monitoring quinone reductase induction in cultured murine hepatoma cells as the biological assay, we have isolated and identified (–)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane [$\text{CH}_3\text{—SO—}(\text{CH}_2)_4\text{—NCS}$, sulforaphane] as a major and very potent phase II enzyme inducer in SAGA broccoli (*Brassica oleracea italica*). Sulforaphane is a monofunctional inducer, like other anticarcinogenic isothiocyanates, and induces phase II enzymes selectively without the induction of aryl hydrocarbon receptor-dependent cytochromes P-450 (phase I enzymes). To elucidate the structural features responsible for the high inducer potency of sulforaphane, we synthesized racemic sulforaphane and analogues differing in the oxidation state of sulfur and the number of methylene groups: $\text{CH}_3\text{—SO}_m\text{—}(\text{CH}_2)_n\text{—NCS}$, where $m = 0, 1, \text{ or } 2$ and $n = 3, 4, \text{ or } 5$, and measured their inducer potencies in murine hepatoma cells. Sulforaphane is the most potent inducer, and the presence of oxygen on sulfur enhances potency. Sulforaphane and its sulfide and sulfone analogues induced both quinone reductase and glutathione transferase activities in several mouse tissues. The induction of detoxication enzymes by sulforaphane may be a significant component of the anticarcinogenic action of broccoli.

Individuals who consume large amounts of green and yellow vegetables have a lower risk of developing cancer (1–3). Feeding of such vegetables to rodents also protects against chemical carcinogenesis (4, 5), and it results in the induction in many tissues of phase II[§] enzymes—e.g., quinone reductase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] and glutathione *S*-transferases (EC 2.5.1.18) (11–17). Although much evidence suggests that induction of these enzymes is a major mechanism responsible for this protection (18–20), the precise role of enzyme induction in protection of humans requires clarification. The preceding report (21) shows that measurement of QR activity in Hepa 1c1c7 murine hepatoma cells provides a rapid, reliable, and convenient index of phase II enzyme inducer activity in vegetables. Using this assay (21–24), we found that cruciferous vegetables (broccoli, cauliflower, mustard, cress, brussels sprouts) were a rich source of inducer activity. We chose to investi-

gate broccoli (*Brassica oleracea italica*) specifically because it is consumed in substantial quantities by Western societies and has been shown to contain abundant phase II enzyme inducer activity (21). In this paper we describe the isolation and identification of a potent major phase II enzyme inducer from broccoli.

MATERIALS AND METHODS

Source of Vegetables and Preparation of Extracts. SAGA broccoli was grown by Andrew Ayer (Maine Packers, Caribou, ME). SAGA is synonymous with Mariner broccoli (Petoseed, Arroyo Grande, CA) and was adapted for growing in Maine by Smith, Ayer, Goughan, and Arrow. The broccoli was harvested when ripe, frozen immediately, shipped to our laboratory in dry ice, and stored at -20°C until processed.

For preliminary survey of inducer activity in broccoli samples, florets were homogenized with 2 vol of water at 4°C , and the resultant soups were lyophilized to give powders, which were stored at -20°C . Portions (400 mg) of these powders were extracted for 6 hr with 14 ml of acetonitrile in glass-stoppered vessels on a wrist-action shaker at 4°C . The extracts were filtered through a sintered glass funnel and evaporated to dryness in a rotating evaporator ($<40^\circ\text{C}$). The residues were dissolved or suspended in 100 μl of dimethyl formamide and assayed for inducer activity.

Assay of Inducer Activity. Inducer potency for QR was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (21, 24). The cells (10,000 per well) were grown for 24 hr and then exposed to inducer for 48 hr. Usually 20 μl of the solutions to be assayed (in acetonitrile or dimethyl formamide) was added to 10.0 ml of medium and 2-fold serial dilutions were used for the microtiter plates. The final organic solvent concentration was always less than 0.2% by volume. One unit of inducer activity is defined as the amount that when added to a single microtiter well (containing 150 μl of medium) doubles the QR specific activity. The inducer potency of compounds of known structure has been determined in the above system also, and it is expressed as

Abbreviations: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2]; CD value, the concentration of a compound required to double the quinone reductase specific activity in Hepa 1c1c7 murine hepatoma cells.

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[§]Enzymes of xenobiotic metabolism belong to two families (6): (i) phase I enzymes (e.g., cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (7); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and play primarily a detoxication role (8). QR is considered a phase II enzyme because it serves protective functions (9), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that regulate glutathione transferases (10).

the concentration required to double (CD value) the QR activity.

The inductions of QR and glutathione transferase activities in mouse organs were studied according to a standard protocol (25).

Synthesis of Compounds. (*R,S*)-Sulforaphane (CAS 4478-93-7) was prepared according to Schmid and Karrer (26) except that gaseous thiomethanol was replaced by sodium thiomethoxide. The sulfide analogues, $\text{CH}_3\text{—S—(CH}_2)_n\text{—NCS}$, where n is 4 [erucin (CAS 4430-36-8)] or 5 [berteroin (CAS 4430-42-6)] were prepared as described (27), and the three-carbon analogue [iberiverin (CAS 505-79-3)] was prepared from phthalimidopropyl bromide (26). IR spectra of all three sulfide analogues showed strong absorptions near 2150 cm^{-1} , characteristic of isothiocyanates. ^1H NMR spectra of these compounds show sharp singlets at $\delta\ 2.10\text{ ppm}$ ($\text{CH}_3\text{—S}$ group). The sulfoxide analogues where n is 3 [iberin (CAS 505-44-2)] or 5 [alyssin (CAS 646-23-1)] were prepared by the same method as sulforaphane. IR spectra of these compounds showed strong absorptions near 2100 cm^{-1} , assigned to the —NCS group. ^1H NMR spectra also showed sharp singlets around $\delta\ 2.5\text{ ppm}$, consistent with the presence of the $\text{CH}_3\text{—SO}$ group. The sulfone analogues, $\text{CH}_3\text{—SO}_2\text{—(CH}_2)_n\text{—NCS}$, where n is 3 [cheirolin (CAS 505-34-0)], 4 [erysolin (CAS 504-84-7)], or 5 (unreported) were prepared by known methods (28). ^1H NMR ($\delta\approx 2.9\text{ ppm}$, for $\text{CH}_3\text{—SO}_2\text{—}$) and IR spectra of these compounds were consistent with the structures. Every analogue except 1-isothiocyanato-5-methylsulfonylpentane [$\text{CH}_3\text{—SO}_2\text{—(CH}_2)_5\text{—NCS}$] has been isolated from plants (29).

RESULTS

Isolation of Inducer Activity. We selected SAGA broccoli for study because acetonitrile extracts of lyophilized homogenates of this variety were especially rich in inducer

activity (62,500 units per g) in comparison with other vegetables (21). Fractionation of acetonitrile extracts of SAGA broccoli by preparative reverse-phase HPLC (Fig. 1) with a water/methanol solvent gradient resulted in recovery of 70–90% of the applied inducer activity in the chromatographic fractions. Surprisingly, the majority (about 65–80% in several chromatographies) of the recovered activity was associated with a single and relatively sharp peak [fractions 18–23; eluted at 64–71% (vol/vol) methanol]. This HPLC procedure was therefore adopted as the first step of the larger-scale isolation of inducer activity.

Lyophilized SAGA broccoli was extracted three times with acetonitrile (35 ml/g) for 6 hr each at 4°C . The pooled extracts were filtered and evaporated to dryness under reduced pressure on a rotating evaporator ($<40^\circ\text{C}$). About 1 g of residue from 640 g of fresh broccoli (64 g of lyophilized powder) contained 3.6×10^6 units of inducer activity. The residue was mixed thoroughly with 120 ml of methanol/water (25/75, vol/vol) and the insoluble fraction was discarded. Although not all of the residue obtained from the extraction was soluble in aqueous methanol, the solvent partition procedure resulted in substantial purification without significant loss of inducer activity. Portions of the extract were dried in a vacuum centrifuge and dissolved in small volumes of dimethyl formamide (0.75–1.0 ml per 50 mg of residue), and 50-mg portions were subjected to HPLC (nine runs) as described in the legend of Fig. 1. Fractions 18–23 from all runs were pooled, evaporated to dryness, applied in acetonitrile to five preparative silica TLC plates ($100 \times 200 \times 0.25\text{ mm}$), and developed with acetonitrile, which was run to the top of each plate three times. Four major fluorescence-quenching components were resolved, and nearly all (99%) of the inducer activity migrated at $R_f\ 0.4$. The active bands were eluted with acetonitrile, pooled, and fractionated by two runs on a second preparative reverse-phase HPLC in a water/

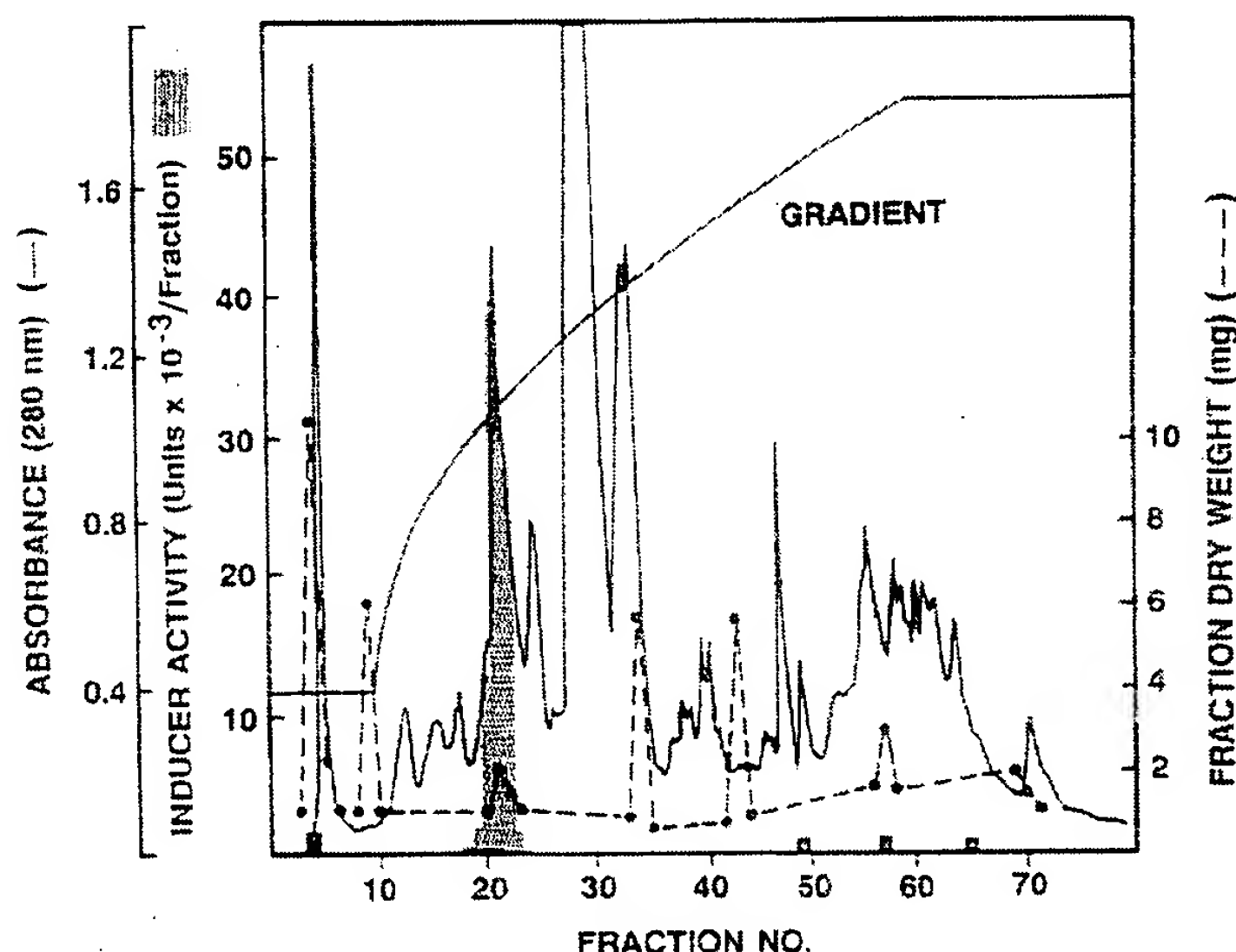
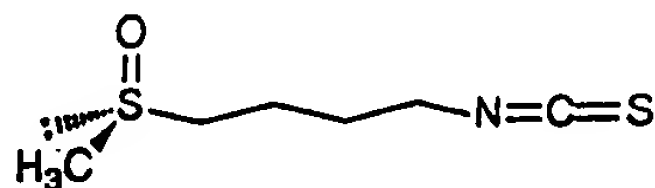


FIG. 1. Reverse-phase HPLC of acetonitrile extract of SAGA broccoli showing the distribution of absorbance at 280 nm, total inducer activity (units per fraction), and dry weight of each fraction. Lyophilized SAGA broccoli floret powder (16 g) was extracted three times (for 6 hr each) with 560-ml portions of acetonitrile on a shaker at 4°C . The extracts were filtered and evaporated to dryness on a rotating evaporator ($<40^\circ\text{C}$). The residue (202 mg) was suspended in 3.0 ml of methanol and filtered successively through 0.45- and 0.22- μm porosity filters. The insoluble material was discarded. The filtrate was assayed for total inducer activity, and a 0.75-ml (50.5-mg) aliquot of the methanol extract was subjected to HPLC on a reverse-phase preparative column (Whatman; Partisil 10 ODS-2; $50 \times 1.0\text{ cm}$) equilibrated with methanol/water (30/70, vol/vol), eluted at a rate of 3.0 ml/min, and 6.0-ml fractions were collected. Elution solvent: 30 ml of initial solvent, followed by 330 ml of a convex gradient (Waters Gradient program 5) to 100% methanol, and then by 90 ml of 100% methanol. The fractions were evaporated on a vacuum centrifuge (Savant Speed-Vac Concentrator), and the residues were weighed, redissolved in 0.1 ml of dimethyl formamide, and assayed for inducer activity. The activity applied (0.75 ml = 104,000 units) was recovered principally in fractions 18–23 (84,600 units, 81%), and minor amounts of activity were found in fractions 4, 49, 57, and 65. The total recovery of inducer activity in all fractions was 90% of that applied to the column.

acetonitrile gradient (Fig. 2). Ultraviolet absorption and inducer activity were eluted in a sharp coincident peak (at 66% acetonitrile) that contained all of the activity applied to the column. Evaporation (<40°C) of the active fractions gave 8.9 mg of a slightly yellow liquid, which contained 558,000 inducer units (overall yield 15%) and migrated as a single band on TLC.

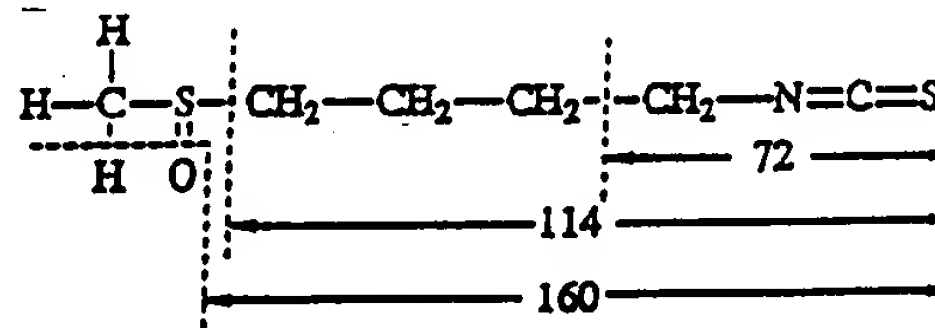
Identification of Inducer. The identity of the inducer was established by spectroscopic methods and confirmed by chemical synthesis. It is (–)-1-isothiocyanato-(4*R*)-(methylsulfinyl)butane, known as sulforaphane or sulphoraphane (CAS 4478-93-7):



Sulforaphane has been synthesized (26) and isolated from leaves of hoary cress (30) and from other plants (31), and the absolute configuration has been assigned (32). The closely related olefin sulforaphene [4-isothiocyanato-(1*R*)-(methylsulfinyl)-1-(*E*)-butene (CAS 2404-46-8)] has been isolated from radish seeds and other plants (33, 34) and has also been synthesized (35, 36).

The following evidence establishes that (*R*)-sulforaphane is the inducer isolated from broccoli. UV spectrum (H₂O): λ_{\max} 238 nm, ϵ_{238} 910 M⁻¹cm⁻¹; addition of NaOH (0.1 M) blue-shifted (λ_{\max} 226 nm) and intensified (ϵ_{226} 15,300 M⁻¹cm⁻¹) this absorption band, consistent with the behavior of isothiocyanates (37). IR (Fourier transform, neat): strong absorptions at 2179 and 2108 cm⁻¹ and also at 1350 cm⁻¹, characteristic of isothiocyanates (27). ¹H NMR (400 MHz,

C²HCl₃): δ 3.60 (t, 2H, $J = 6.1$ Hz, —CH₂—NCS), 2.80–2.66 (m, 2H, —CH₂—SO—), 2.60 (s, 3H, CH₃—SO—), and 1.99–1.86 ppm (m, 4H, —CH₂CH₂—). ¹³C NMR (400 MHz, C²HCl₃): δ 53.5, 44.6, 38.7, 29.0, and 20.1 ppm. Mass spectrometry (fast atom bombardment; thioglycerol matrix) gave prominent peaks at 178 (M + H)⁺ and 355 (M₂ + H)⁺. Electron impact mass spectrometry gave a small molecular ion (M⁺) at 177, and chemical ionization mass spectrometry gave a small molecular ion (M + H)⁺ at 178 and prominent fragment ions with masses of 160, 114, and 72, consistent with the following fragmentation:



Precise masses of molecular and fragment ions obtained by electron impact mass spectrometry were 177.0286 (calculated for C₆H₁₁NOS₂, 177.0283), 160.0257 (calculated for C₆H₁₀NS₂, 160.0255), and 71.9909 (calculated for C₂H₂NS₁, 71.9908). In addition, for the mass 160 fragment, the peaks at 161 (M + 1) and 162 (M + 2) were 8.43% (calculated, 8.44%) and 9.45% (calculated, 10.2%), respectively, of the parent ion. Similarly, for the mass 72 fragment, the peaks at 73 (M + 1) and 74 (M + 2) were 3.42% (calculated, 3.32%) and 5.23% (calculated, 4.44%), respectively, of the parent ion. Hence the isotope compositions corrected for the natural isotope abundance (of ¹³C, ¹⁵N, ³³S, and ³⁴S) were consistent with the relative intensities of the M + 1 and M + 2 ions of both fragments. The optical rotation of the isolated material was [α]_D²³ –63.6° ($c = 0.5$, CH₂Cl₂), thus establishing that the product is largely, if not exclusively, the (–)-(*R*) enantiomer (literature [α]_D –79°, –73.2°, –66°; refs. 26, 30, and 38, respectively). The spectroscopic properties of synthetic (*R,S*)-sulforaphane were identical to those of the isolated product.

Relation of Structure to Inducer Activity Among Sulforaphane Analogues. To define the structural features of sulforaphane (chirality, state of oxidation of sulfur of the thiomethyl group, number of methylene bridging groups) important for inducer activity, we synthesized (*R,S*)-sulforaphane and the following analogues and measured their inducer potencies: CH₃—S—(CH₂)_{*n*}—N=C=S ($n = 3, 4$, or 5); CH₃—SO—(CH₂)_{*n*}—N=C=S ($n = 3$ or 5); and CH₃—SO₂—(CH₂)_{*n*}—N=C=S ($n = 3, 4$, or 5).

Induction of QR in Murine Hepatoma Cells. The chirality of the sulfoxide does not affect inducer potency, since isolated (*R*)-sulforaphane and synthetic (*R,S*)-sulforaphane gave closely similar CD values of 0.4–0.8 μ M. Sulforaphane is therefore the most potent monofunctional (see below) inducer that has been identified (19, 20). Both (*R*)- and (*R,S*)-sulforaphane were relatively noncytotoxic: the concentrations required to depress cell growth to one-half were 18 μ M.

Sulforaphane and the corresponding sulfone (erysolin) were equipotent as inducers of QR, whereas the corresponding sulfide (erucin) was about one-third as active (Table 1). Oxidation of the side-chain sulfide to sulfoxide or sulfone enhanced inducer potency, and compounds with 4 or 5 methylene groups in the bridge linking CH₃S— and —N=C=S were more potent than those with 3 methylene groups (Table 1).

Mutants of Hepa 1c1c7 cells defective in the Ah (aryl hydrocarbon) receptor or expression of cytochrome P-450IA1 can distinguish monofunctional inducers (which induce phase II enzymes selectively) from bifunctional in-

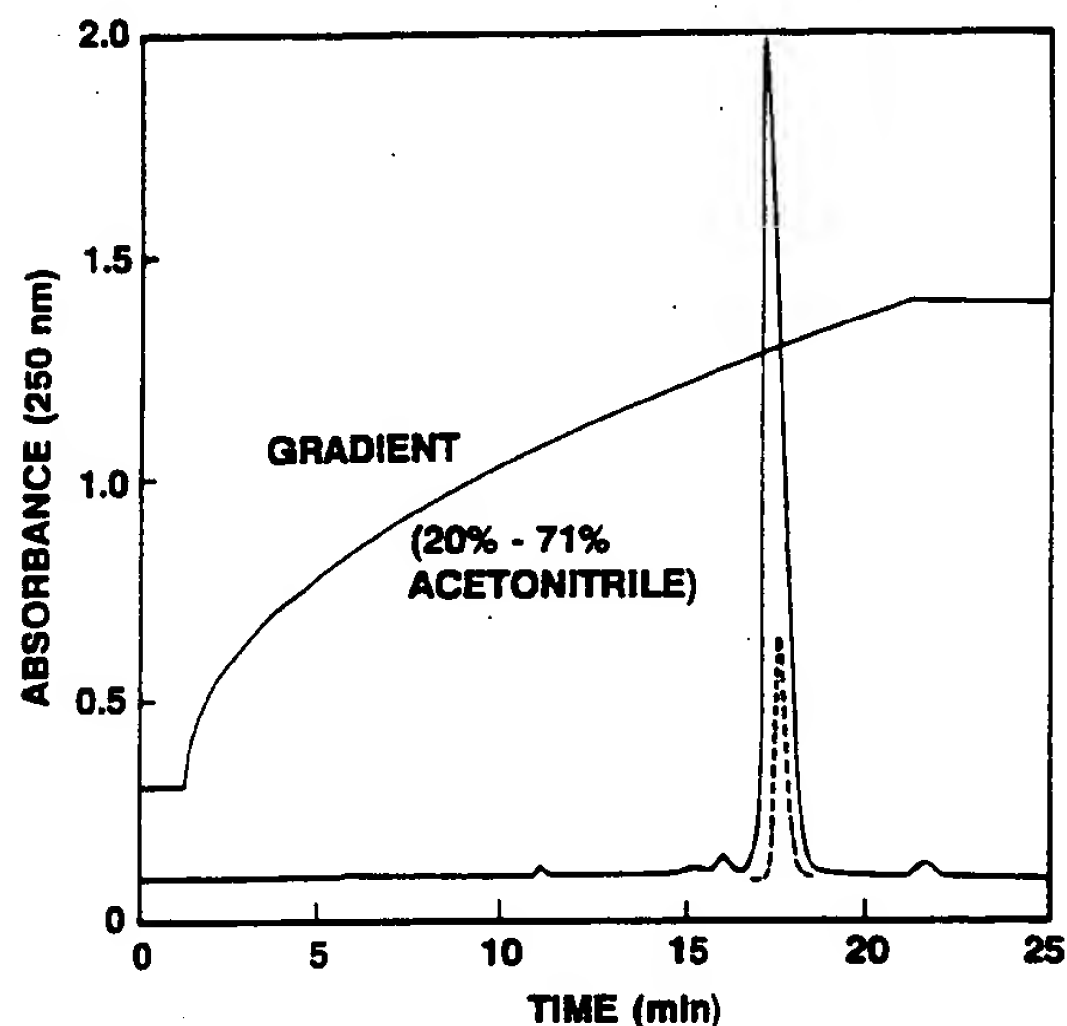


FIG. 2. Second reverse-phase preparative HPLC of enzyme inducer activity from SAGA broccoli. The active inducer bands obtained from two or three preparative silica TLC plates (see text) were combined, eluted with acetonitrile, filtered twice through 0.22- μ m porosity filters, and evaporated to dryness on a vacuum centrifuge. The residue was dissolved in 0.5 ml of acetonitrile and applied to a reverse-phase preparative HPLC column (Whatman; Partisil ODS-2; 50 \times 1.0 cm), which was developed with a convex gradient (Waters Gradient program 5) of acetonitrile/water from 20:80% to 71:29% (vol/vol) at a flow rate of 3.0 ml/min during a 20-min period. The eluate from 17.0 to 19.0 min was collected as a pool and assayed for inducer activity; 99% of the inducer activity was recovered in this pool. The elution position of (*R,S*)-sulforaphane is shown (—).

Table 1. Potency of induction of QR in Hepa 1c1c7 cells by sulforaphane and analogues

Compound	CD value, μ M		
	<i>n</i> = 3	<i>n</i> = 4	<i>n</i> = 5
$\text{CH}_3\text{—S—(CH}_2\text{)}_n\text{—N=C=S}$	3.5 (Iberverin)	2.3 (Erucin)	1.7 (Berteroin)
$\text{CH}_3\text{—S—(CH}_2\text{)}_n\text{—N=C=S}$	2.4 (Iberin)	0.4–0.8 (Sulforaphane)	0.95 (Alyssin)
$\text{CH}_3\text{—S(=O)—(CH}_2\text{)}_n\text{—N=C=S}$	1.3 (Cheirolin)	0.82 (Erysolin)	0.98

Trivial names are given in parentheses. See Kjær (29).

ducers (which elevate both phase I and II enzymes) (39, 40). When sulforaphane was tested with the BP^ccl mutant (41) (defective in transport of the liganded Ah receptor to the nucleus), and the cl mutant (42) (which synthesizes inactive cytochrome P-450IA1), induction of QR was normal (data not shown). Sulforaphane is, therefore, like benzyl isothiocyanate, a monofunctional inducer (40) and is unlikely to elevate activities of cytochromes P-450 that could activate carcinogens.

Induction of QR and Glutathione Transferase Activities in Mice. When synthetic (*R,S*)-sulforaphane, erysolin, and erucin were administered to female CD-1 mice by gavage (25), induction of QR and glutathione transferase activities was observed in the cytosols of several organs (Table 2). Sulforaphane and erucin (in daily doses of 15 μ mol for 5 days) raised both enzyme activities 1.6- to 3.1-fold in liver, forestomach, glandular stomach, and mucosa of proximal small intestine, and to a lesser degree in lung. The sulfone (erysolin) was more toxic, but even 5- μ mol daily doses for 5 days elevated the specific activities of these enzymes in some tissues examined. We therefore conclude that sulforaphane and its analogues not only induce QR in Hepa 1c1c7 murine hepatoma cells but also induce both QR and glutathione transferase activities in a number of murine organs.

DISCUSSION

Two considerations prompt the belief that sulforaphane is a major and probably the principal inducer of phase II enzymes present in extracts of SAGA broccoli. First, high yields of

inducer activity were obtained at each step of the isolation, and even in the first HPLC (Fig. 1) more than 60% of the inducer activity was contained in a single chromatographic peak, the biological activity of which could not be subfractionated. Second, when a totally independent method of isolation of inducer activity by high-vacuum sublimation of lyophilized broccoli (5 μ m Hg pressure, 60–165°C, condenser at –15°C) was used, nearly all the isolated inducer activity was found in the methanol-soluble portion of the sublimate. Moreover, on HPLC (Fig. 2) this sublimed material gave rise to only a single isothiocyanate-containing fraction, which on TLC comigrated with authentic sulforaphane and after further purification by TLC provided a high yield of sulforaphane characterized unequivocally by NMR.

The finding that the majority of the inducer activity of SAGA broccoli probably resides in a single chemical entity, an isothiocyanate, is of considerable interest. Isothiocyanates (mustard oils) and their glucosinolate precursors are widely distributed in higher plants and are especially prevalent among cruciferous vegetables (29). Sulforaphane has been identified in species of *Brassica*, *Eruca*, and *Iberis* (29, 31).

Isothiocyanates have been shown to block chemical carcinogenesis. In rats, 1-naphthyl isothiocyanate suppressed hepatoma formation by 3-methylcholanthrene, 2-acetylaminofluorene, diethylnitrosamine, *m*-toluenediamine, and azo dyes (43–46). In mice, benzyl isothiocyanate blocked the neoplastic effects of diethylnitrosamine or benzo[*a*]pyrene on lung and forestomach (47, 48), and a variety of phenylalkyl isothiocyanates reduced the pulmonary carcinogenicity of

Table 2. Induction of QR and glutathione *S*-transferase (GST) in mouse tissues by sulforaphane and analogues

Inducer	Dose, μ mol per mouse per day	Enzyme	Ratio of specific activities (treated/control)				
			Liver	Forestomach	Glandular stomach	Proximal small intestine	Lung
$\text{CH}_3\text{—S—(CH}_2\text{)}_4\text{—NCS}$ Erucin	15	QR	2.19 \pm 0.06	1.64 \pm 0.18*	1.72 \pm 0.11	3.10 \pm 0.20	1.66 \pm 0.13
		GST	1.86 \pm 0.08	2.51 \pm 0.11	2.07 \pm 0.08	3.00 \pm 0.21	1.41 \pm 0.11*
$\text{CH}_3\text{—S—(CH}_2\text{)}_4\text{—NCS}$ Sulforaphane	15	QR	2.45 \pm 0.07	1.70 \pm 0.18*	2.35 \pm 0.06	2.34 \pm 0.19	1.37 \pm 0.14*
		GST	1.86 \pm 0.08	1.98 \pm 0.08	2.97 \pm 0.08	2.13 \pm 0.20	1.17 \pm 0.09†
$\text{CH}_3\text{—S(=O)—(CH}_2\text{)}_4\text{—NCS}$ Erysolin	5	QR	1.62 \pm 0.09	1.05 \pm 0.21†	1.57 \pm 0.08†	1.22 \pm 0.20†	1.00 \pm 0.11†
		GST	1.08 \pm 0.11†	1.45 \pm 0.15†	1.94 \pm 0.10†	0.87 \pm 0.20†	1.09 \pm 0.13†

The compounds were administered to 6-week-old female CD-1 mice (4 or 5 mice per group) by gavage in indicated single daily doses in 0.1 ml of Emulphor EL 620P (GAF, Linden, NJ) for 5 days. Cytosols were prepared from the tissues 24 hr after the last treatment and assayed for enzyme activities (glutathione *S*-transferase was measured with 1-chloro-2,4-dinitrobenzene). The specific activities (nmol·min^{–1}·mg^{–1} \pm SEM) of organs of vehicle-treated control mice were as follows. Liver: QR, 47 \pm 0.70; GST, 1014 \pm 69. Forestomach: QR, 1038 \pm 155; GST, 1182 \pm 74. Glandular stomach: QR, 3274 \pm 85; GST, 1092 \pm 81. Small intestine: QR, 664 \pm 119; GST, 1372 \pm 266. Lung: QR, 54 \pm 5.8; GST, 439 \pm 34. Data are presented as mean \pm SEM. All ratios were significantly different from 1.0 with *P* < 0.01, except for *, *P* < 0.05, and †, *P* > 0.05.

the tobacco-derived carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (49, 50). The anticarcinogenic effects of previously studied isothiocyanates may be related to their capacity to induce phase II enzymes, which are involved in the metabolism of carcinogens (51–57).

It will be important to establish whether the alterations of drug metabolism observed in humans and rodents after the ingestion of cruciferous vegetables (58, 59) can be ascribed to their content of sulforaphane. The finding that this isothiocyanate is a major monofunctional inducer of phase II enzymes in broccoli also provides the possibility of clarifying the relationship between enzyme induction and chemoprotection.

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Detection of inducers of enzymes that protect against carcinogens

(cancer/chemoprotection/enzyme induction/quinone reductase/vegetables)

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ABSTRACT Dietary composition is a major determinant of cancer risk in humans and experimental animals. Major and minor components of the diet may enhance or suppress the development of malignancy. Many dietary constituents also modify the metabolism of carcinogens by induction of enzymes involved in xenobiotic metabolism, and this is one well-established mechanism for modulating the risk of cancer. We have developed a simple system for rapid detection and measurement of the induction of enzymes that detoxify carcinogens (phase II enzymes), based on the direct assay of the activity of quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase; EC 1.6.99.2] in murine hepatoma cells grown in microtiter plate wells. Survey of extracts of a variety of commonly consumed, organically grown vegetables for quinone reductase inducer activity identified crucifers (and particularly those of the genus *Brassica*) as singularly rich sources. It is therefore of interest that high consumption of these types of vegetables has been correlated with decreased cancer risk in humans. The assay system also measures toxicity, which was unrelated to inducer potency among the vegetable extracts examined. By use of mutant hepatoma cells (defective in regulation of certain cytochrome P-450 enzymes) selective (monofunctional) inducers of protective phase II enzymes can be distinguished from (bifunctional) inducers that also elevate cytochromes P-450 (phase I enzymes) and thereby pose the risk of carcinogen activation. The assay system therefore permits not only rapid detection of inducers of anticarcinogenic enzymes in the human diet but also elucidation of effects of storage and processing on inducer activities.

Extrinsic factors, including personal life-styles, play a major role in the development of most human malignancies (1-3). Cigarette smoking and consumption of alcohol, exposure to synthetic and naturally occurring carcinogens, radiation, drugs, infectious agents, and reproductive and behavioral practices are now widely recognized as important contributors to the etiology of cancer. But perhaps most surprising is the inference that normal human diets play causative roles in more than one-third (and possibly even two-thirds) of human neoplasia (1-3). Our food contains not only numerous mutagens and carcinogens but also a variety of chemicals that block carcinogenesis in animal models (4-11). Furthermore, carcinogens can even protect against their own toxic and neoplastic effects or those of other carcinogens—i.e., carcinogens may act as anticarcinogens (12-14). Clearly, dietary modifications modulate cancer risk in various ways: for instance, through changes in caloric intake, by altering the consumption of nutritive and nonnutritive major components, and by providing exposure to numerous minor chemicals that may be genotoxic or protective (4-7, 9-11, 15-19). Rational recommendations for modifying human diets to

reduce the risk of cancer require identification of dietary carcinogens and chemoprotectors, even though interactions among such factors in the etiology of cancer are complex (20). Whereas extensive efforts have been made to identify dietary carcinogens and mutagens (4-6), chemoprotective components have received far less attention. This paper describes a method for detecting and identifying anticarcinogenic components in human diets.

✱ Since a major mechanism regulating neoplasia is the balance between phase I enzymes, which activate carcinogens, and phase II enzymes[‡] (25, 26), which detoxify them, we have developed a cell culture system for simple and rapid detection of dietary components that enhance phase II detoxication enzymes. With this procedure we surveyed extracts of a variety of vegetables for their ability to induce such protective enzymes. In the accompanying paper (27) we describe use of this method to isolate and identify a major inducer of protective enzymes from broccoli.

We chose vegetables as sources of inducers of detoxication enzymes for the following reasons. First, numerous epidemiological studies suggest that high consumption of yellow and green vegetables, especially those of the family Cruciferae (mustards) and the genus *Brassica* (cauliflower, cress, brussels sprouts, cabbage, broccoli), reduces the risk of developing cancer of various organs (28-34). Moreover, administration of vegetables or of some of their chemical components to rodents also protects against chemical carcinogenesis (9-11, 35). Second, well-documented evidence established that feeding of certain vegetables (e.g., brussels sprouts and cabbage) induces both phase I and phase II enzymes in animal tissues (36-44) and stimulates the metabolism of drugs in humans (36, 45, 46). The elevations of enzymes that metabolize xenobiotics may be highly relevant to the protective effects of vegetables, since relatively modest dietary changes not only affected the metabolism of drugs (44) but also modified the ability of carcinogens to cause tumors in rodents (15-19, 47-49).

Several lines of evidence provide compelling support for the proposition that induction of enzymes of xenobiotic

Abbreviation: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2].

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‡Enzymes of xenobiotic metabolism belong to two families: (i) phase I enzymes (cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (21); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and serve primarily a detoxication role (22). Quinone reductase (QR) is considered a phase II enzyme because it has protective functions (23), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that control glutathione transferase (24).

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metabolism, and particularly phase II enzymes, results in protection against the toxic and neoplastic effects of carcinogens (25, 26): (i) Many seemingly unrelated compounds (including phenolic antioxidants, coumarins, cinnamates, 1,2-dithiole-3-thiones, isothiocyanates, lactones, thiocarbamates) can protect rodents against the effects of carcinogens under conditions that invariably evoke the induction of phase II enzymes in many tissues. Indeed, novel anticarcinogens have been isolated and identified solely on the basis of their ability to induce phase II enzymes (50, 51). (ii) Such anticarcinogens alter the metabolism of carcinogens and decrease the formation of mutagenic metabolites (52–54). (iii) Chemoprotection requires protein synthesis and is most effective if it precedes carcinogen challenge (12–14). (iv) Inducers of anticarcinogenic enzymes protect against a wide variety of structurally dissimilar carcinogens, suggesting the involvement of mechanisms that are not structurally fastidious, such as xenobiotic metabolism. (v) The enzymes that are elevated, e.g., glutathione transferases, quinone reduc-

tase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2], UDP-glucuronosyltransferases, protect against the toxicities of electrophiles such as ultimate carcinogens. (vi) Cells in which glutathione transferases are elevated (by development of resistance to alkylating chemotherapeutic agents or by transfection with cloned enzymes) show decreased susceptibility to the toxicity of carcinogenic electrophiles and reduced formation of DNA adducts (55–57).

Resolution of the issue whether the anticarcinogenic effects of vegetables are mediated through the induction of enzymes of xenobiotic metabolism requires the systematic bioassay of these plants for inducer activity. Since measurement of enzyme induction in animals is laborious and expensive, we developed a simple screening procedure in which the specific activity of QR, a phase II enzyme, is measured in Hepa 1c1c7 murine hepatoma cells (58, 59). The feasibility of measuring inducer activity directly in cells grown in 96-well microtiter plates has simplified and accelerated the procedure (60), and the use of heat- and charcoal-treated serum in-

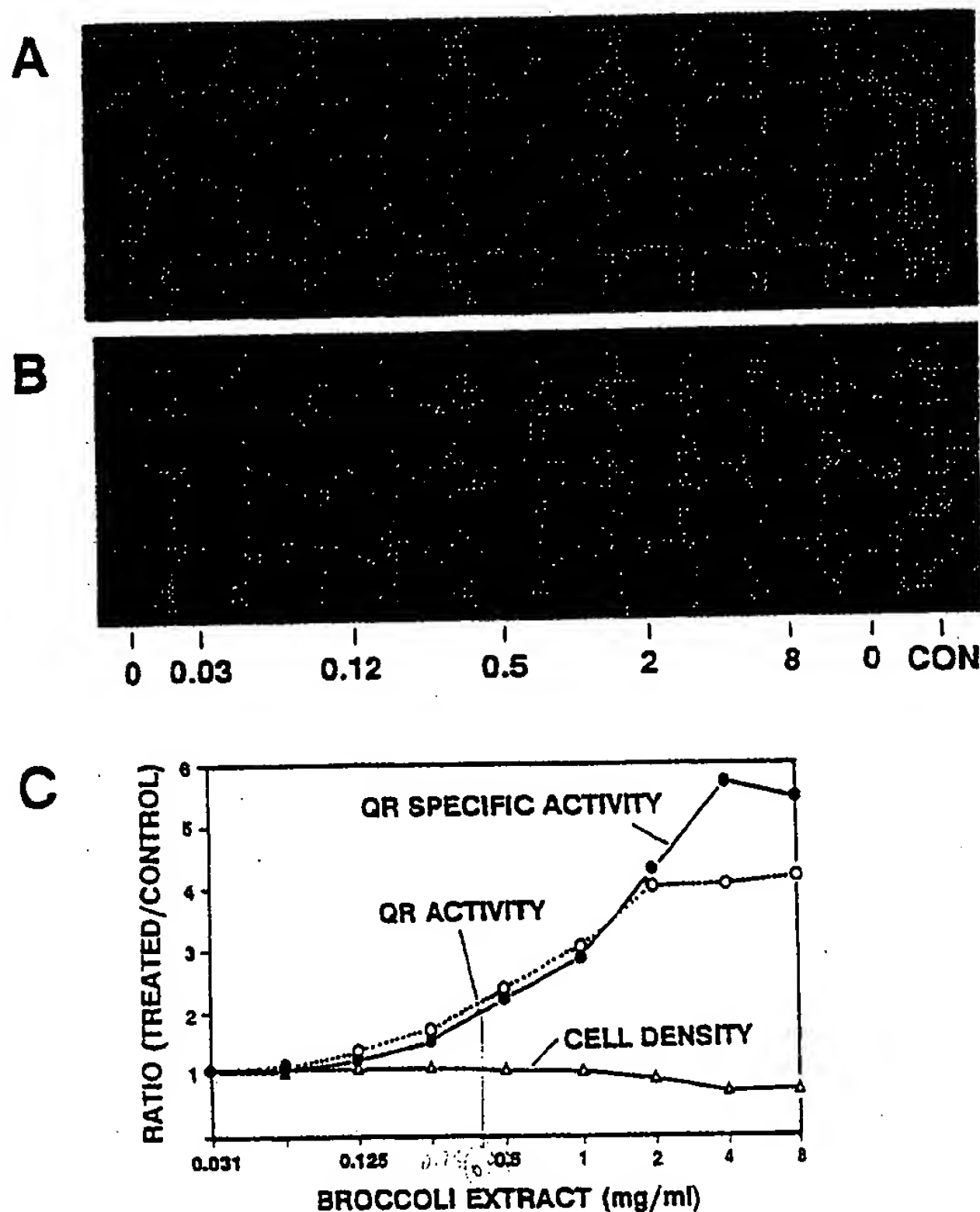


FIG. 1. Induction of QR in murine hepatoma cells by extracts of broccoli. (A and B) Photographs of sections of 96-well microtiter plates showing the induction of QR (A) and the cell density (B). (C) Graph showing the analysis of absorbances obtained from the plates. The assays were carried out on Hepa 1c1c7 murine hepatoma cells grown in microtiter plate wells and induced with serial 2-fold dilutions of acetonitrile extracts of lyophilized broccoli (Effie May). Details of the procedures are given in *Materials and Methods*. (A) The QR activities were measured in cell lysates by reduction of a tetrazolium dye. Note that the color (blue-brown) increases in intensity with the concentration of extract, indicating QR induction. (B) A parallel plate treated with the same dilutions of broccoli extract. The cells were stained with crystal violet. Note that there is a slight decrease in cell density at the highest concentrations (4–8 mg/ml), indicating mild cytotoxicity. (C) Graphical analysis of optical density information obtained from the above plates scanned at 610 nm (QR assay) and 490 nm (crystal violet assay) related to control wells that received the equivalent volume of acetonitrile only (0.2%). The total and specific activities of QR and the cell densities, expressed as ratios (treated/control), are shown on the ordinate. The concentrations of broccoli extract, shown below the designated microtiter plate wells and on the abscissa of the graph, are expressed as the amount of extract obtained from a given dry weight of broccoli (mg) added to each ml of culture medium (0–8 mg/ml). The QR activity and crystal violet density are related to cells that did not receive inducer. The columns of wells designated 0 mg/ml received no broccoli extract. CON designates the wells that contained no cells and served as the optical controls.

creased its sensitivity (61). These cells respond to nearly all compounds that induce phase II enzymes (e.g., QR and glutathione transferases) in rodents, and conversely induction of QR in these cells is a reliable predictor of inducer activity in various rodent organs *in vivo* (27, 61–63).

MATERIALS AND METHODS

Sources of Vegetables and Preparation of Extracts. All vegetables were grown under organic conditions without pesticides or artificial fertilizers that might contain enzyme inducers. They were stored at -20°C after arrival in our laboratory, although the intervening storage history of some vegetables is not known. Vegetables were homogenized with 2 vol of cold water in a Waring Blendor for 2 min at 4°C . The resultant soups were lyophilized to give dry powders, which were stored at -20°C . Portions (400 mg) of these powders were extracted for 6–24 hr with 14 ml of acetonitrile by shaking in glass vessels at 4°C . The extracts were filtered through $0.45\ \mu\text{m}$ porosity organic solvent-resistant filters and evaporated to dryness either in a vacuum centrifuge (Speed-Vac; Savant) or on a rotating evaporator ($<40^{\circ}\text{C}$). The residues were dissolved or suspended in $100\ \mu\text{l}$ of acetonitrile.

Assay of Inducer Potency. Inducer activity was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (60, 61). Duplicate plates were prepared. In a typical experiment 10,000 Hepa 1c1c7 cells were introduced into each well initially, grown for 24 hr, and then induced for 48 hr by exposure to medium containing serial dilutions of the extracts (or compounds) to be assayed. Usually $20\ \mu\text{l}$ of the acetonitrile solutions to be assayed were added to $10.0\ \text{ml}$ of medium and 2-fold serial dilutions were made in the microtiter plates so that the final volume in each well was $150\ \mu\text{l}$ and the organic solvent concentration was 0.2%. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) was measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and the cell density was determined in the second plate by staining with crystal violet. Quantitative information on specific activity of QR, the inducer potency, and the cytotoxicity of the extract or compound tested is obtained by computer analysis of the absorbances (see Fig. 1). One unit of inducer activity is defined as the amount that when added to a single microtiter well doubled the QR specific activity.

RESULTS AND DISCUSSION

Fig. 1 illustrates the measurement of inducer potency of extracts of organically grown broccoli (Effie May variety). The specific activities of QR were raised nearly 6-fold at the highest extract concentrations tested, at which less than 20% cytotoxicity was observed. The inductions obtained with broccoli (Fig. 1) and with other vegetable extracts (Fig. 2) were proportional to the quantity of extract added over a reasonably wide range. The toxicities of these extracts were modest and were unrelated to their inducer potencies (Fig. 2).

Extracts of a series of organically grown vegetables cultivated under a variety of conditions showed large differences in inducer potencies (Table 1). Because the dry weight content of the vegetables varied considerably, from 3.6% for a sample of bok choy to 26.5% for red leaf lettuce (mean 10% for 24 vegetables) (Table 1), we express the inducer activity of an extract in terms of the dry weight of vegetable yielding, upon extraction under standardized conditions, a given amount of inducer activity. This provides a measure of inducer potency, expressed as units per g of dry vegetable weight (see *Materials and Methods* for definition of unit). Although many vegetable extracts induced QR, certain families were consistently more potent inducers. For example,

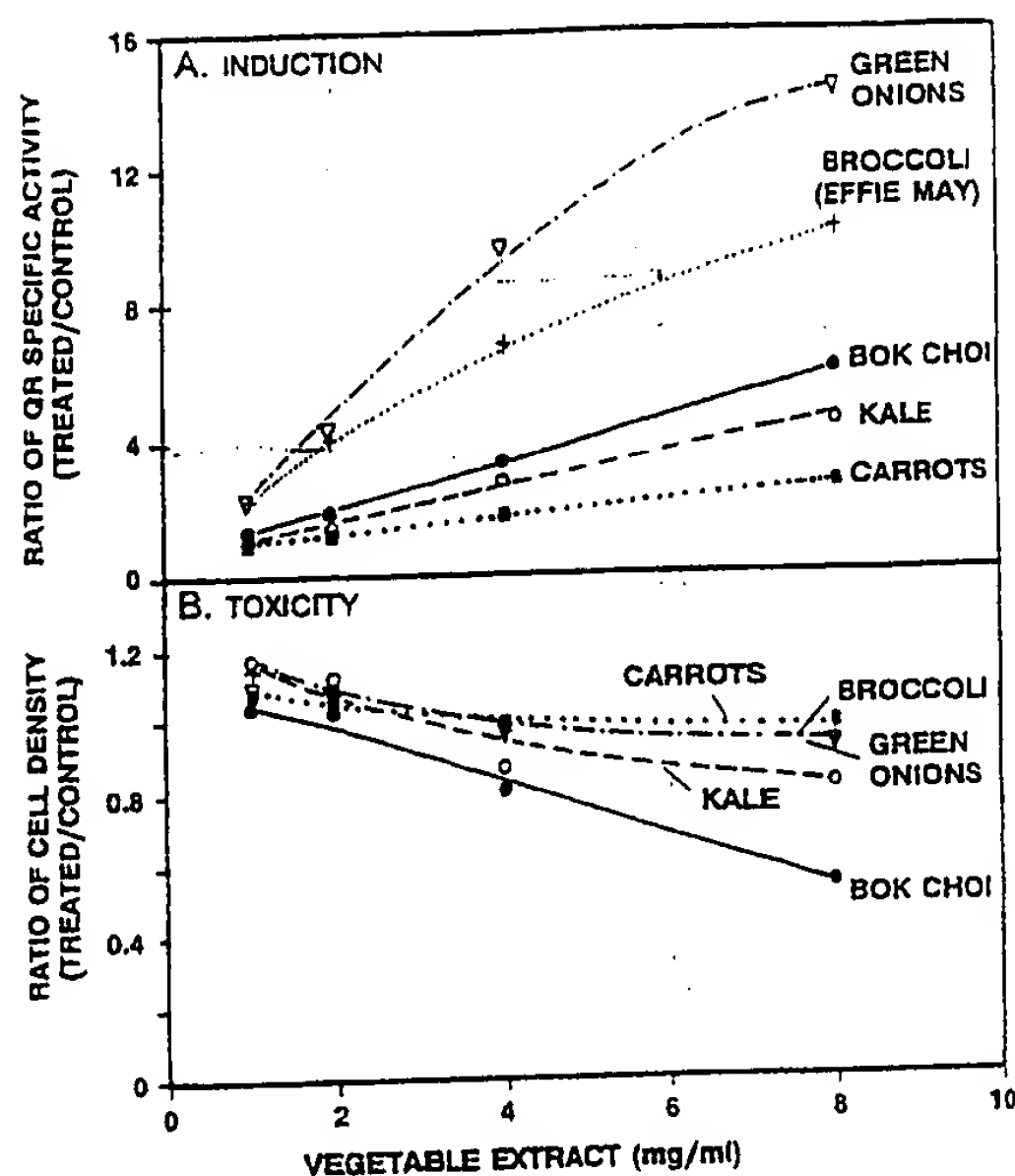


FIG. 2. Potency of induction of QR and toxicity of acetonitrile extracts obtained from five organically grown lyophilized vegetables (green onion, broccoli, bok choy, kale, and carrots) measured by the microtiter plate assay in Hepa 1c1c7 murine hepatoma cells. The extracts were prepared and assayed as described in Fig. 1 legend and *Materials and Methods*. The concentrations of extracts are expressed as the amount of extract per ml of culture medium derived from the indicated weight of dried vegetable. (A) Ratio of the specific activities of QR of treated to control cells. (B) Relative cell densities as determined by crystal violet staining measured at 490 nm. Note that the inductions are reasonably proportional to the amount of extract at lower induction ratios and that the inducer potencies and toxicities (which do not exceed 20% except in the case of bok choy) are not correlated.

whereas extracts of several Cruciferae had potent inducer activity, extracts of Solanaceae (peppers, potatoes, tomatoes) had low inducer activity. Of the 24 vegetables examined only 6 showed detectable toxicity; the others were nontoxic at the highest concentrations tested. Thus cytotoxicity of 20% was observed for red leaf lettuce at 8.0 mg/ml, for beets, cauliflower, and bok choy at 4.0 mg/ml, and for leeks and ginger at 2.0 mg/ml.

Cytotoxicity measurements are important because phase II enzyme inducers may be toxic and/or carcinogenic. Moreover, by use of mutant Hepa cells defective in aryl hydrocarbon receptor or cytochrome P-450 function (27, 63, 64), our assay system can distinguish *monofunctional* inducers (which elevate phase II enzymes selectively), from *bifunctional* inducers (which elevate both phase I and phase II enzymes) (63). Such information is crucial for identification of chemoprotective enzyme inducers for potential use in humans. Ideally such inducers should be monofunctional, because elevated activities of phase I enzymes may lead to carcinogen activation.

Since some crucifers (broccoli, brussels sprouts, cauliflower, cabbage) are consumed in substantial quantities in Western diets and are believed to protect against cancer, we examined the relation of inducer potency to variety, strain, location of growth, time of sowing, and time of harvest (Table 2). Although systematic examination of these factors under field conditions would require extensive studies over several

Table 1. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of representative samples of various vegetable families and species

Vegetable		Dry weight, %	Potency of QR induction, units*/g
Family	Species/variety		
Chenopodiaceae	Beets	17.3	<833
	Spinach	6.8	1,280
Compositae	Red leaf lettuce	26.5	3,030
Cruciferae	Cauliflower	9.8	2,220
	Bok choy	3.6	3,170
	Broccoli (Effie May)	10.2	16,700
	Broccoli (Winchester)	8.0	2,380
	Green cabbage	9.4	1,550
	Kale	9.2	2,220
	Radish	3.7	1,040
Cucurbitaceae	Zucchini	5.5	<833
Leguminosae	Green beans	6.7	2,150
	Sugar snap peas	14.5	<833
Liliaceae	Asparagus	5.6	1,110
	Green onions	5.1	22,200
	Leeks	8.3	2,780
Rosaceae	Apple	13.1	Inactive
Solanaceae	Green peppers	6.4	Inactive
	Red potatoes	15.7	Inactive
	Sweet potatoes	20.2	Inactive
	Tomatoes	6.2	<833
Umbelliferae	Carrots	10.8	1,230
	Celery	4.5	1,630
Zingiberaceae	Ginger	13.1	4,440

*One unit of inducer activity is defined as the amount of inducer required to double the QR specific activity of Hepa 1c1c7 cells growing in a microtiter well containing 150 μ l of medium. An entry of <833 units/g indicates that at the highest concentration tested (extract from 1.2 mg of dry vegetable/150- μ l medium) the QR specific activity was significantly elevated but not doubled. Inactive indicates less than 20% elevation of QR specific activity at highest concentration tested: extract from 1.2 mg of dry weight per 150 μ l of medium.

8mg/ml = 1 unit.
1.2mg = 1 unit.

years of cultivation, it was important to determine whether such variables significantly affected the inducer activity. Except for a sample of kohlrabi, Cruciferae belonging to the species *Brassica oleracea* consistently and potently induced QR (Table 2), with broccoli and brussels sprouts generally the most potent inducers. The inductive capacity of most crucifers appears to be independent of geographic location of growth and time of harvest, although late sowing may have enhanced modestly the potency of the induction. On the basis of these results a particular variety of broccoli (SAGA) was selected for isolation and identification of monofunctional inducer activity as described in the accompanying paper (27).

In summary, epidemiological studies point to the inverse relationship between vegetable consumption and the risk of epithelial cancer, and they suggest a practical approach to achieving protection by emphasizing that the typical Western diet is low in fruits and vegetables (20). A striking but perhaps not surprising conclusion is that the microtiter plate assay for induction of QR identifies the same vegetables (crucifers) that display protective properties *in vivo* (9-11, 28-35). It is critical to our understanding of the relationship of diet to cancer, however, that we assess dietary constituents not only for their abilities to induce anticarcinogenic enzymes but also for their toxic and carcinogenic properties. The simple and rapid assay can also determine the toxicity of extracts and, by use of appropriate mutant cells, distinguish monofunctional inducers from less desirable bifunctional ones. Moreover, the assay of phase II enzymes makes possible further detailed analysis of the effects of treatment of vegetables (e.g.,

Table 2. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of various cruciferous vegetables of the species *Brassica oleracea*

Vegetable (<i>B. oleracea</i> subspecies and variety)	Dry weight, %	Potency of QR induction, units/g
Broccoli (<i>B. oleracea italica</i>)		
Florets		
Emperor	9.6	6,670
Emperor (late sowing)	9.9	22,200
Green Valiant	10.4	16,700
Green Valiant (late sowing)	8.8	16,700
SAGA (older sample)	6.8	4,170
SAGA (younger sample)	8.0	11,100
SAGA (late sowing)	10.1	33,300
Violet Queen	9.6	3,700
Leaves only		
Emperor	7.0	5,560
SAGA (older sample)	9.4	3,030
SAGA (younger sample)	10.0	16,700
Violet Queen	10.6	7,410
Brussels sprouts (<i>B. oleracea gemnifera</i>)		
Jade Cross E	10.8	11,100
Oliver	15.6	6,060
Teal	14.0	11,100
Green cabbage (<i>B. oleracea capitata</i>)		
Nagoda 50	4.6	2,560
Perfect Ball	7.6	5,560
Primax	5.6	2,080
Red cabbage (<i>B. oleracea capitata</i>)		
Lasso Red	11.2	13,300
Ruby Perfection	7.0	4,760
Cauliflower (<i>B. oleracea botrytis</i>)		
Florets		
Andes	8.8	5,560
Montano	8.0	3,700
Leaves only		
Montano	7.4	3,330
Snow Crown	6.6	2,780
Kale (<i>B. oleracea acephala</i>)		
Konserva	10.4	3,170
Winterbor	8.4	4,760
Winterbor (late sowing)	15.4	16,700
Kohlrabi (<i>B. oleracea gongyloides</i>)		
Capri	5.2	1,330
Kolpak (late sowing)	6.0	1,590

The assays were performed as described in the text and legend to Table 1.

growth, storage, and cooking conditions) that might enhance or depress such induction, and also more definitive examination of the relationship of induction to chemoprotection.

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The use of primary hepatocyte cultures for the evaluation of chemoprotective agents

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Keywords: chemoprotection, cytochrome P450, glutathione *S*-transferase, hepatocytes, human, *in vitro* preparation, rat

Abstract

Primary cultures of human and rat hepatocytes are widely used in pharmacotoxicological research. This model presents the advantages of retaining liver function for at least a few days, expressing both phase 1 and phase 2 enzymes, and responding to inducers. Recently we made use of primary hepatocytes to investigate the effects of chemoprotective agents on drug-metabolizing enzyme expression and activities. The treatment of rat and human hepatocytes with two chemoprotective agents, oltipraz (a synthetic derivative of 1,2-dithiole-3-thione) and sulforaphane (an isothiocyanate found in broccoli), clearly demonstrated that both of these compounds are inducers of glutathione transferases and transient inhibitors of cytochrome P450, suggesting that these two compounds could exert their chemoprotective effects by both reducing the formation of reactive metabolites of chemicals and enhancing their inactivation.

Abbreviations: AFB₁, aflatoxin B₁; AFBO, *exo*-aflatoxin-8,9-epoxide; CYP, cytochrome P450; GST, glutathione *S*-transferase

Introduction

The liver is the chief organ involved in the metabolism of xenobiotics. It plays a major role in conversion of lipophilic compounds to hydrophilic metabolites that can be readily excreted. The metabolism of chemicals usually involves two enzymatic stages commonly referred to as phase 1 and phase 2. Phase 1 metabolism is ensured mostly by cytochrome P450 (CYP) monooxygenases, resulting in an oxidized metabolite that is further conjugated or reduced (phase 2). Conjugation is catalyzed by UDP-glucuronyltransferases, sulfotrans-

ferases and glutathione transferases (GSTs) and reduction by epoxide hydrolase and quinone reductase. However, metabolism of a number of chemicals can lead to the formation of toxic metabolites and/or of reactive oxygen species. Indeed, activation may result in the formation of electrophilic metabolites that bind to lipids and proteins, leading to injury to cells, or to DNA, leading, in the absence of repair, to mutation and cancer. In humans, six CYPs (1A1, 1A2, 1B1, 2A6, 2E1, and 3A4) are involved in the conversion of promutagens to mutagens. Among the endogenous detoxifying systems, GSTs play a critical role in protection

against electrophiles and products of oxidative stress (Hayes and Pulford, 1995). The principal GSTs are soluble and form a supergene family containing many enzymes that belong to four main multigene families, alpha, mu, pi and theta. In the past years, a number of studies have emphasized their contribution to cancer chemoprevention (Kensler et al., 1987; Hayes et al., 1991).

Chemoprevention can be obtained by the administration of one or more chemical entities either as individual drugs or as naturally occurring constituents of the diet (Morse and Stoner, 1993). Absolute classification of the chemoprotective compounds is difficult owing to the fact that the precise mechanism(s) of action are not known for many compounds (Wattenberg, 1985). Blocking agents, also called antiinitiating agents, are of peculiar importance; they can act as inhibitors or inducers of CYPs, inducers of phase 2 enzymes, particularly GSTs, scavengers of electrophiles, or inducers of DNA repair (Morse and Stoner, 1993) (Figure 1). Among the most potent chemoprotective agents are isothiocyanates (Hecht, 1995) and dithiolethiones

(Kensler et al., 1987), either the naturally occurring molecules or synthetic derivatives. These compounds have been shown to inhibit the formation of tumors induced by various chemicals in different tissues. Isothiocyanates appear to be capable of both inhibiting CYPs and inducing GSTs (Zhang and Talalay, 1994; Conoway et al., 1996), while dithiolethiones have been reported to be potent inducers of phase 2 enzymes (Kensler et al., 1987; Hayes et al., 1991; Primanio et al., 1995).

Most observations on chemoprevention have come from studies on animal models. Except for direct-acting carcinogens, genotoxic carcinogens must first be metabolically activated to electrophilic forms that can damage DNA. However, the rates and the routes of xenobiotic metabolism are often widely different between species, particularly when comparisons involve laboratory animals and man, making questionable extrapolation to humans of the data obtained in animals. Since studies on chemoprevention, with the exception of epidemiological ones, are ethically not acceptable in humans, the use of *in vitro* model systems represents an appropriate experimental approach to the subject.

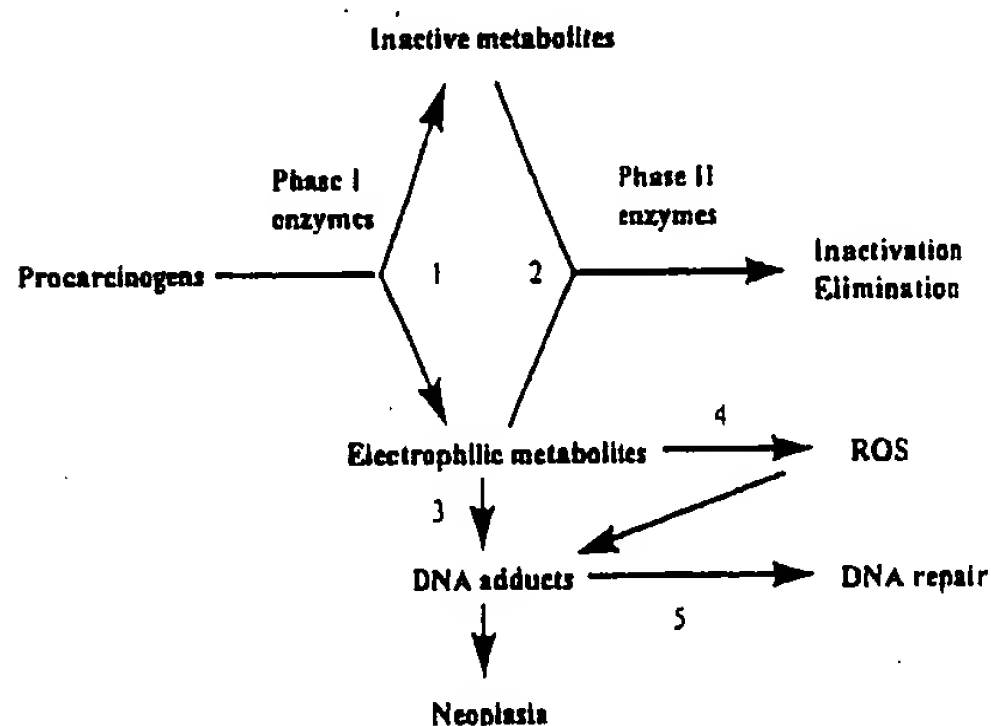


Figure 1. The anticarcinogenic effects of chemoprotective agents can be mediated by different mechanisms: (1) suppression of carcinogen activation by CYPs; (2) induction of phase 2 enzymes, such as GSTs, which detoxify residual electrophilic metabolites; (3) scavenging of electrophilic metabolites; (4) scavenging of reactive oxygen species (ROS); (5) increase of DNA repair enzymes.

The choice of the *in vitro* models for chemoprevention studies

The different *in vitro* liver preparations include the isolated perfused organ, tissue slices, isolated hepatocytes either in suspension or in primary culture, liver cell lines, subcellular fractions, and genetically engineered cells expressing one or more liver-specific functions. The advantages and limitations of these different *in vitro* model systems are summarized in Table 1. Obviously, primary hepatocyte cultures appear to represent the most powerful model. Indeed, a number of studies have shown that adult hepatocytes can be maintained functional for at least a few days in

Table 1. Advantages and limitations of *in vitro* liver preparations

Model	Advantages	Limitations
Isolated perfused liver	<ul style="list-style-type: none"> Functions close to those of the <i>in vivo</i> organ (all enzyme equipment preserved) Lobular structure preserved Functional bile canaliculi Collection of bile possible Short-term kinetic studies (extraction) 	<ul style="list-style-type: none"> Short term viability (2–3 h) Study of one compound only Bile excretion decreased after 1–3 h No study on human liver Suitable only for liver of small animals
Liver slices	<ul style="list-style-type: none"> Lobular structure preserved (all enzyme equipment preserved) Selective intralobular effects detectable Studies on human liver possible Studies on several compounds at different concentrations 	<ul style="list-style-type: none"> Viability: 6 h – 2 days No collection of bile possible Not all the cells preserved similarly (interassay variability)
Isolated hepatocytes ^a	<ul style="list-style-type: none"> Availability from whole livers or wedge biopsies Functions close to those of <i>in vivo</i> hepatocytes Studies on several compounds at different concentrations Cryopreservation Interspecies studies Representative of the different lobular subpopulations 	<ul style="list-style-type: none"> Short-term viability (2–4 h) No bile canaliculus
Primary hepatocyte cocultures ^a	<ul style="list-style-type: none"> Functions expressed for several days in certain conditions Induction/inhibition of drug-metabolizing enzymes Interspecies studies 	<ul style="list-style-type: none"> Early phenotypic changes Altered bile canaliculi
Liver cell lines	<ul style="list-style-type: none"> Unlimited cell number Some functions preserved 	<ul style="list-style-type: none"> Various drug-enzyme activities lost or decreased Genotype instability
Subcellular fractions S9000 fraction	<ul style="list-style-type: none"> Drug enzyme activities preserved 	<ul style="list-style-type: none"> Short-term studies Cofactors required for activity
Microsomes	<ul style="list-style-type: none"> Drug enzyme activities preserved Production of metabolites for structural analysis 	<ul style="list-style-type: none"> Short-term studies No cytosolic phase 2 enzyme reactions Cofactors required for activity
Genetically engineered cells	<ul style="list-style-type: none"> One or more human enzymes expressed Available only for CYPs Unlimited cell numbers 	<ul style="list-style-type: none"> Use for specific purposes only No physiological levels of enzymes

^aOther cell types can also be isolated, cultured, and even cocultured with hepatocytes: e.g., Kupffer, endothelial, stellate, and bile duct cells.

primary culture, human hepatocytes being more stable than their rodent counterparts.

The duration of survival and prolonged expression of liver-specific functions *in vitro*

are dependent upon culture conditions. Three groups of factors are influential: soluble factors (i.e., composition of the medium), extracellular matrix components, and cell-cell interactions

(i.e., pericellular environment). Cocultures of hepatocytes with primitive biliary cells, a model first described in our laboratory in 1983 (Guguen-Guillouzo et al., 1983) appears to remain the most powerful *in vitro* system (Blaauboer et al., 1994). Several reviews have been published on primary hepatocyte cultures (Guillouzo et al., 1990; Rogiers, 1993; Guillouzo, 1995a).

Freshly isolated hepatocytes can be transiently preserved by hypothermia or cryopreserved for months or years. When stored at 4°C, either in Leibovitz medium or in University of Wisconsin solution, rat hepatocytes can survive for 1 or 2 days. Parenchymal cells from various species can be cryopreserved. However, cell viability and attachment are reduced and drug-metabolizing enzyme activities are more or less impaired. Recently, we found that, when entrapped in an alginate gel, isolated rat hepatocytes show only limited loss of viability and alteration of functions after cryopreservation (Guyomard et al., 1996).

A good qualitative *in vivo/in vitro* correlation in metabolic profiles has been observed for most chemicals studied. Moreover, the cells remain responsive to inducers of both phase 1 and phase 2 enzymes. In our laboratory we have shown that in human hepatocytes CYPs are inducible by specific inducers such as 3-methylcholanthrene, rifampicin, and phenobarbital (Morel et al., 1990) and alpha class GST, and to a lesser extent mu class GST, by phenobarbital and oltipraz, a synthetic derivative of 1,2-dithiole-3-thione (Morel et al., 1993).

However, it must be borne in mind that *in vitro* hepatocytes do not fully mimic the *in vivo* situation even when sophisticated culture conditions are used, some functions being more unstable than others, for example, sulfotransferase activities. Moreover, marked inter-individual functional variations that can occur between the human liver cell populations must be considered. Thus variations of 40-fold in

ethoxyresorufin *O*-deethylase activity (supported by CYP1A; $n=19$) and of 10-fold in GST activity using 1-chloro-2,4-dinitrobenzene as a substrate ($n=14$) have been found in early human hepatocyte cultures (Guillouzo and Chesné, 1996).

Chemoprotection in primary hepatocyte cultures

The chemoprotective properties of naturally occurring substances and their synthetic analogs such as dithiolethiones and isothiocyanates have been investigated mainly in animal models. 1,2-Dithiole-3-thione exhibits a wide range of antioxidant, chemotherapeutic and chemoprotective effects. The synthetic derivative oltipraz has been shown to inhibit tumorigenesis in various tissues including liver, breast, colon, and urinary bladder in rodents (Kensler and Helzlsouer, 1995). This agent was found to be a very potent protective agent against aflatoxin B₁ (AFB₁)-induced hepatocarcinogenesis in the rat and its effect has been associated with induction of alpha class GST, particularly GSTA5, an enzyme which is not expressed in adult rat liver (Hayes et al., 1991). AFB₁, a product of the mold *Aspergillus flavus* which infests grains and other foodstuffs stored under warm conditions, is also a human hepatocarcinogen: hepatocellular carcinoma is common in many communities that consume an AFB₁-contaminated diet. In a recent study we questioned whether oltipraz could be so effective in humans since the potential for GST-dependent detoxication of AFB₁ in the human liver is not as great as in the rat liver. Indeed, human liver has about one order of magnitude less GST activity toward the genotoxin *exo*-aflatoxin-8,9-epoxide (AFBO), and no apparent homologue for rat GSTA5. When exposed to oltipraz, human hepatocytes showed an induction of GSTA1 and GSTA2 and, to a lower extent (when expressed),

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GSTM1 (Morel et al., 1993; Langouët et al., 1995). This M1 subunit is inherited in an autosomal fashion and is virtually absent in 50% of the human population. Analysis of AFB₁ metabolism by HPLC showed two main metabolites: AFM₁ (nontoxic), and AFBO conjugated with glutathione. Glutathione conjugates were formed only in human hepatocytes expressing GST mu (M1) (Table 2) suggesting that GSTM1-null individuals could be more susceptible to AFB₁-induced cancer. This observation supports and could explain the study of Liu and colleagues (1991), who showed a role for GSTM1 in modulating the formation of carcinogen-derived adducts. In humans, AFBO formation involves CYP1A2 and CYP3A4 (Ueng et al., 1995). Surprisingly, oltipraz was found to decrease AFM₁ and glutathione conjugates of AFBO as well as overall AFB₁ metabolism in human hepatocytes, indicating that it inhibited CYP1A2 and CYP3A4 (Langouët et al., 1995). This inhibiting effect was confirmed by measuring specific-related enzyme activities in cultured human hepatocytes and by using human recombinant yeast CYP1A2 and CYP3A4. This study with primary human hepatocytes was the first demonstration that oltipraz can act both as a GST inducer and as a CYP inhibitor and suggested that in humans it could be protective against AFB₁ toxicity both by reducing activation and by increasing inactivation.

Such an inhibition of CYPs had not been described before in the rat. This prompted us to carry out a similar study on primary rat hepatocytes and for comparison in rat *in vivo*. The increase of different GST subunits, including GSTA5, was first demonstrated by measuring mRNA, protein and/or enzyme activity levels in primary rat hepatocytes treated with oltipraz (Langouët et al., 1996). As shown in human hepatocytes, this chemoprotective agent also inhibited ethoxyresorufin *O*-deethylase supported by CYP1A and pentoxyresorufin *O*-deethylase supported by CYP2B as

Table 2. Metabolism of aflatoxin B₁ in human hepatocytes

	AFB ₁ (μ mol/L)	AFBSG (μ mol/L)	AFM ₁ (μ mol/L)	Metabolism (%)
GSTM1⁺				
Control	1.17	0.43	0.36	76
Oltipraz	1.91	0.07	<0.01	61
GSTM1⁻				
Control	1.70	<0.01	0.04	65
Oltipraz	2.49	<0.01	<0.01	50

From Langouët et al. (1995)

Hepatocytes expressing GSTM1 (GSTM1⁺) or not (GSTM1⁻) were cultured in the presence or absence of 50 μ mol/L oltipraz for 2 days and were exposed to 5 μ mol/L AFB₁ for 8 h. AFB₁, glutathione conjugates of AFBO (AFBSG), and AFM₁ contained in the culture medium were measured by HPLC.

well as CYPs 2C6, 2C11, and 3A2. This inhibition was transient, being completely reversed within 24 h. A similar inhibition of CYP1A2 was found in rats by measuring metabolites of caffeine given with oltipraz. Indeed, the levels of the three main primary metabolites of caffeine – theobromine, theophylline and paraxanthine – were considerably reduced in oltipraz-treated rats (Langouët et al., 1997). Following transient inhibition of CYP1A and CYP2B, an induction became apparent for these enzymes as soon as 24 h after oltipraz intake. Corresponding mRNAs were found to be increased, suggesting that oltipraz induced at least some CYP genes in addition to genes encoding GSTs and other phase II enzymes. Similar observations have been made by Buetler and colleagues (1995).

Recently, in order to determine whether the effects observed with oltipraz are a more general feature of chemoprotective agents, we studied the effects of the isothiocyanate sulforaphane, a natural compound from broccoli known as a very potent inhibitor of chemically induced carcinogenesis in rodents (Zhang et al., 1992, 1994). We investigated the effects of this isothiocyanate using primary rat and human hepatocytes. As observed with oltipraz,

the results clearly demonstrate that sulforaphane is both an inducer of GST and an inhibitor of CYPs, suggesting that it could also exert its chemoprotective effects by reducing the formation of reactive metabolites of chemicals and enhancing their inactivation (Mahéo et al., submitted).

Conclusion and perspectives

Primary hepatocytes retaining their phase 1 and phase 2 metabolic activities represent a unique model for investigating chemoprotective effects against carcinogens and other toxins in humans and in animals. In the human, drug metabolism can only be followed to a limited degree by analysis of metabolites in the blood and urine. Primary hepatocytes represent a surrogate for analysing the effects of chemoprotective agents on metabolism of toxic compounds. This model allows both kinetic and induction analyses and can be considered as the closest *in vitro* system of the liver *in vivo*.

By using primary hepatocytes we obtained the first evidence of an inhibitory effect of oltipraz of some major CYPs involved in the metabolism of xenobiotics. However, the mechanism of inhibition remains unclear and deserves further study, the transient inhibition could be related to metabolism of oltipraz and its short half-life. Another interesting observation made with rat hepatocytes is the induction of CYPs after 24 h or more of treatment with oltipraz. Whether similar induction of CYPs by oltipraz also occurs in human hepatocytes has not yet been established clearly.

Comparison of results obtained with human and rat hepatocytes leads to interesting conclusions. *In vitro/in vitro* extrapolation of data cannot easily be made. Indeed, CYPs and GST subunits involved in the metabolism of chemicals are frequently different in the two species. Moreover, in humans several CYPs

and GSTs exhibit a genetic polymorphism of expression or inducibility and it must be borne in mind that huge interindividual variations are frequent in most phase 1 and phase 2 enzymes.

However, availability of human liver tissues, particularly isolated hepatocytes, is scarce and unpredictable (Guillouzo, 1995b). It is therefore advisable to use various *in vitro* liver preparations. Regarding the effects of chemoprotective agents, the use of hepatic microsomes and recombinant human CYPs expressed in yeast has proved to be quite valuable for completing studies with human hepatocytes.

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Address for correspondence: Fabrice Morel, INSERM U 456, Unité Détoxication et Réparation Tissulaire, Université de Rennes 1, Faculté de Pharmacie, 2 Avenue du Professor Léon Bernard, 35043 Rennes Cedex, France

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Brovelli et al
Serial No: 09/575,307
Filing Date: May 19, 2000
Examiner: Michael V. Meller
Group Art: 1654
For: ECHINACEA INDUCTION OF PHASE II ENZYMES

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.131

We declare that:

1. We are co-inventors of the patent application identified above.
2. Prior to May 9, 2000, we had completed the invention as described and claimed in the subject application in this country as evidenced by the following:
 - a. the Invention Conception Record and accompanying attachments submitted to the Alticor Legal Department (formerly Amway Corporation) (attached as Exhibit 1);
 - b. Lab notebook pages from one inventor, Kari Truax, (attached as Exhibit 2).
3. The Invention Conception Record and its accompanying figures show that, prior to May 9, 2000, we conceived of and reduced to practice a procedure for inducing phase II enzymes with chloroform-soluble *Echinacea purpurea* fractions as described and claimed in the subject application.
4. Additionally, Kari Truax's lab notebook pages outline the steps she performed to reduce our invention to practice in the Nutrilite Laboratories at 19600 6th Street, Lakeview, CA 92567-8403. These steps conform with Figure 1 and Pages 6-7 of the subject application and further support that, prior to May 9, 2000, we conceived of and reduced to practice, a procedure for inducing phase II enzymes with chloroform-soluble fractions of *Echinacea purpurea* as described and claimed in the subject application.
5. Each of the dates redacted from Exhibits 1 and 2 are prior to May 9, 2000.

6. We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

Ernesto Brovelli, Ph.D

Date _____

Yingqin Li

Date _____

Kari Fitzgerald (maiden name – Truax)

Date _____

Puri David

Date _____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Brovelli et al
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Ernesto Brovelli, Ph.D

Date

Yingqin Li

Date



Kari Fitzgerald (maiden name - Truax)

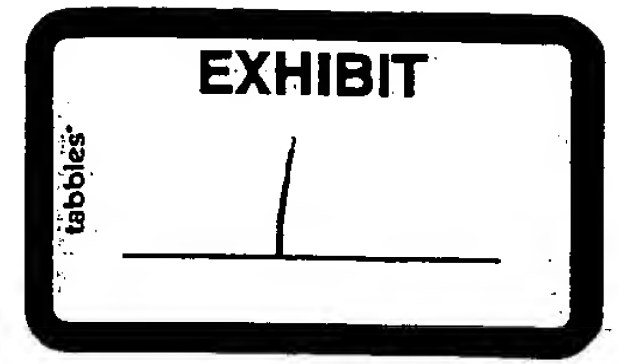
10/8/04

Date

Puri David

Date

**Amway Corporation
Invention Conception Record**



A. Title of your invention.

Phase II Enzyme Induction by *Echinacea* Extract and Its Fractions

B. Describe your invention including specific embodiments and alternatives, ranges and products, and process/apparatus variations. Include photographs, sketches, flow sheets, or other drawings where appropriate.

We have found that certain *Echinacea purpurea* extracts and some of its fractions induce the expression of Phase II enzyme. The extracts found to induce the expression of this enzyme are derived from both the aboveground organs and the roots.

On that basis, experimentation was further refined to determine the level of induction for *Echinacea purpurea* fractions extracted with solvents of different polarity. It was determined that for aboveground organs, it is the petroleum ether fraction that results in the greatest induction of phase II enzyme. For roots, it is the chloroform fraction that brings about maximum induction.

C. Describe what problem was solved and how you solved it. Describe how your invention differs from art you are aware of and describe the advantages of your invention.

To our knowledge, and based on an extensive literature review, there is no previous knowledge about this kind of mechanism in *Echinacea*. The induction of phase II enzymes has extremely important implications for the human body. These enzymes have detoxifying activity of potential carcinogens. In addition to *Echinacea*'s well-documented immunomodulatory functions, this finding can extend the activity of *Echinacea* as a cancer chemopreventive agent.

D. When did you first think of this invention?

On ^{redacted} we thought that the recently developed prototypes might have Phase II enzyme properties and decided to submit them to a bioassay.

E. What records do you have to document when you first thought of this invention?

Laboratory notebook.

F. To whom did you first disclose this invention?

Kha Tran

G. On what date did you make this disclosure?

redacted

H. When did you first do any actual experimental work concerning the invention?

redacted

I. Who has observed the progress of your experimental work?

Rodney Johnson & Kevin Gellenbeck.

J. Are there other ICRs now on file or contemplated, yours or others, that tie in closely with this invention?

No.

K. Has panel or consumer testing been conducted?

Yes _____ No. X If yes, when? Describe the testing.

L. Are there any plans for consumer or panel testing?

Yes _____ No. X If yes, when? give details.

Not in the immediate plans.

M. Give dates and details regarding any samples, sales, information, or publications relating to this invention which have been or will be given to persons outside Amway Corporation.

No information has been disclosed to outside parties.

N. Are you aware of any pertinent literature or patent references? Give details.

None whatsoever.


O. Is commercial use imminent? If yes, indicate the anticipated earliest date of commercial use.

The extracts are currently being manufactured by Nutrilite/Amway, although with the purpose of enhancing the immune system.

This is an extremely novel function for this herb and it would be advantageous to exploit this benefit commercially.

NOTE: IF ANY CHANGES TO THE ABOVE INFORMATION OCCUR,
ADVISE A MEMBER OF THE PATENT REVIEW TEAM IMMEDIATELY.

Inventor Ernesto A. Brovelli

Signed  redacted
month, day, year

Inventor Yong Quian

Signed _____
month, day, year

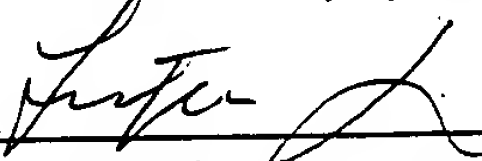
Inventor Puri G. David

Signed Puri G. David redacted
month, day, year

Inventor Kari L. Truax

Signed Kari L. Truax redacted
month, day, year

Inventor Yingqin Li

Signed  redacted
month, day, year

The foregoing signed disclosure was read and understood by me on the date
hereinafter set forth.

Witness 

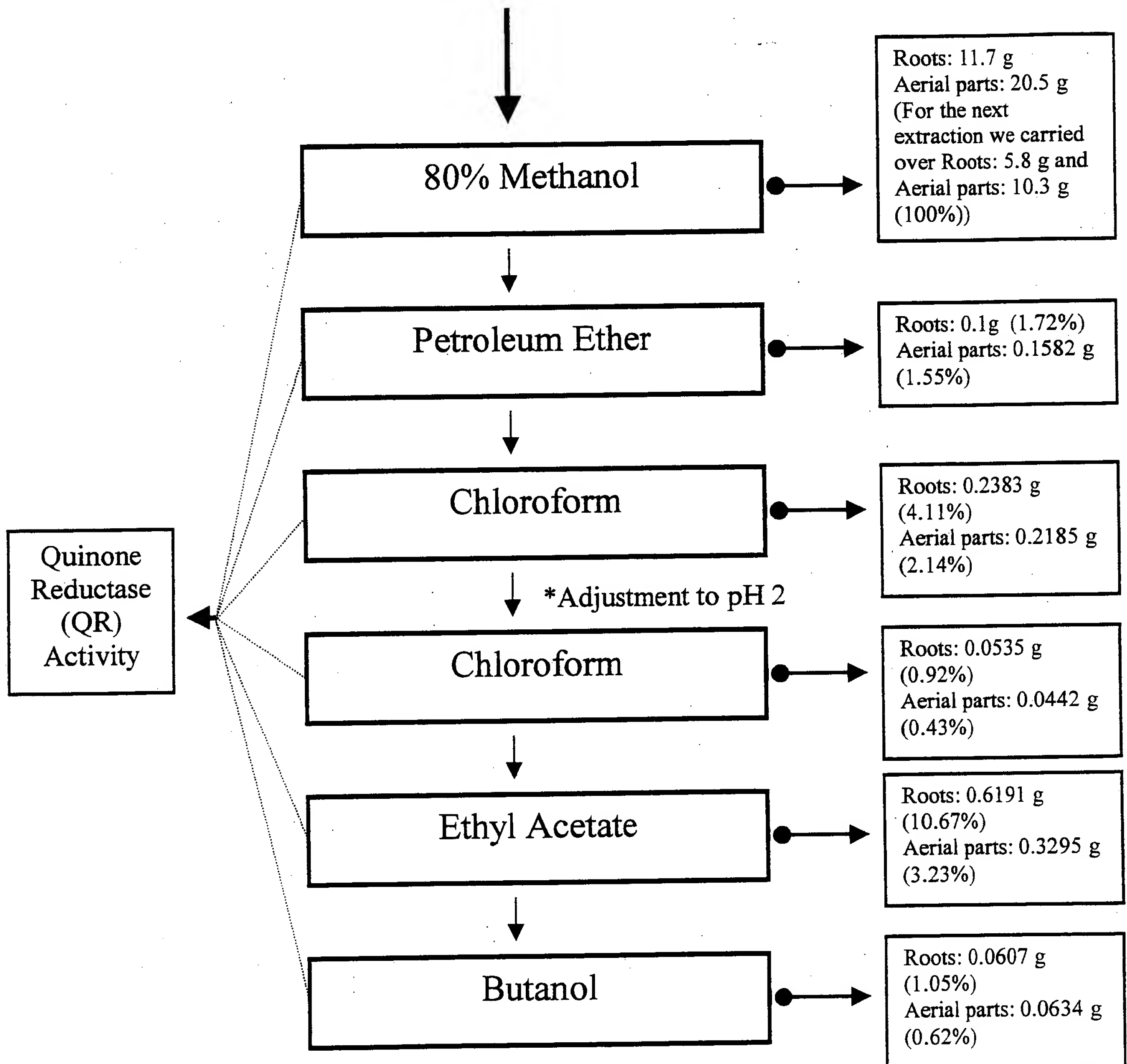
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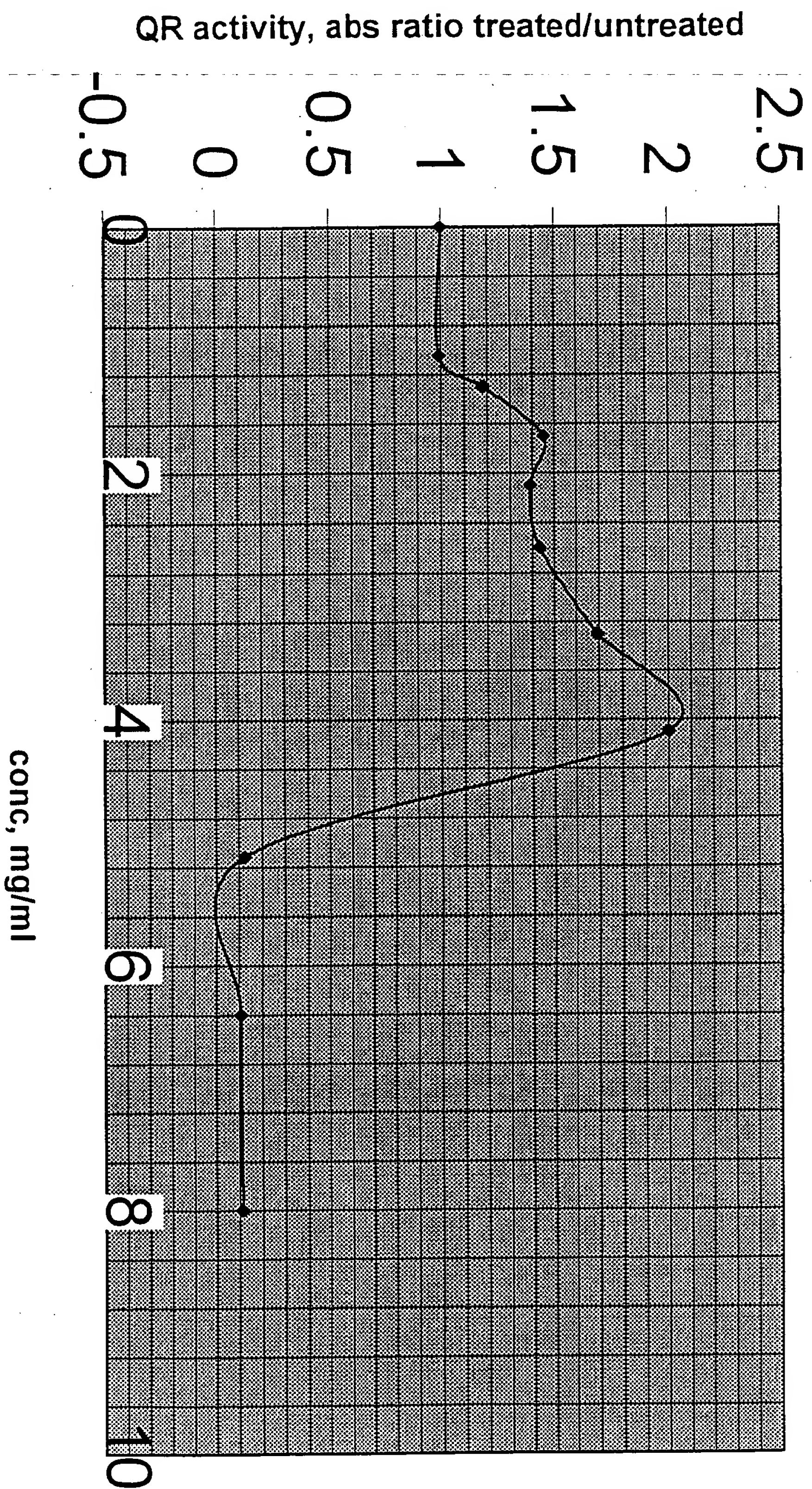
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Extraction Protocol

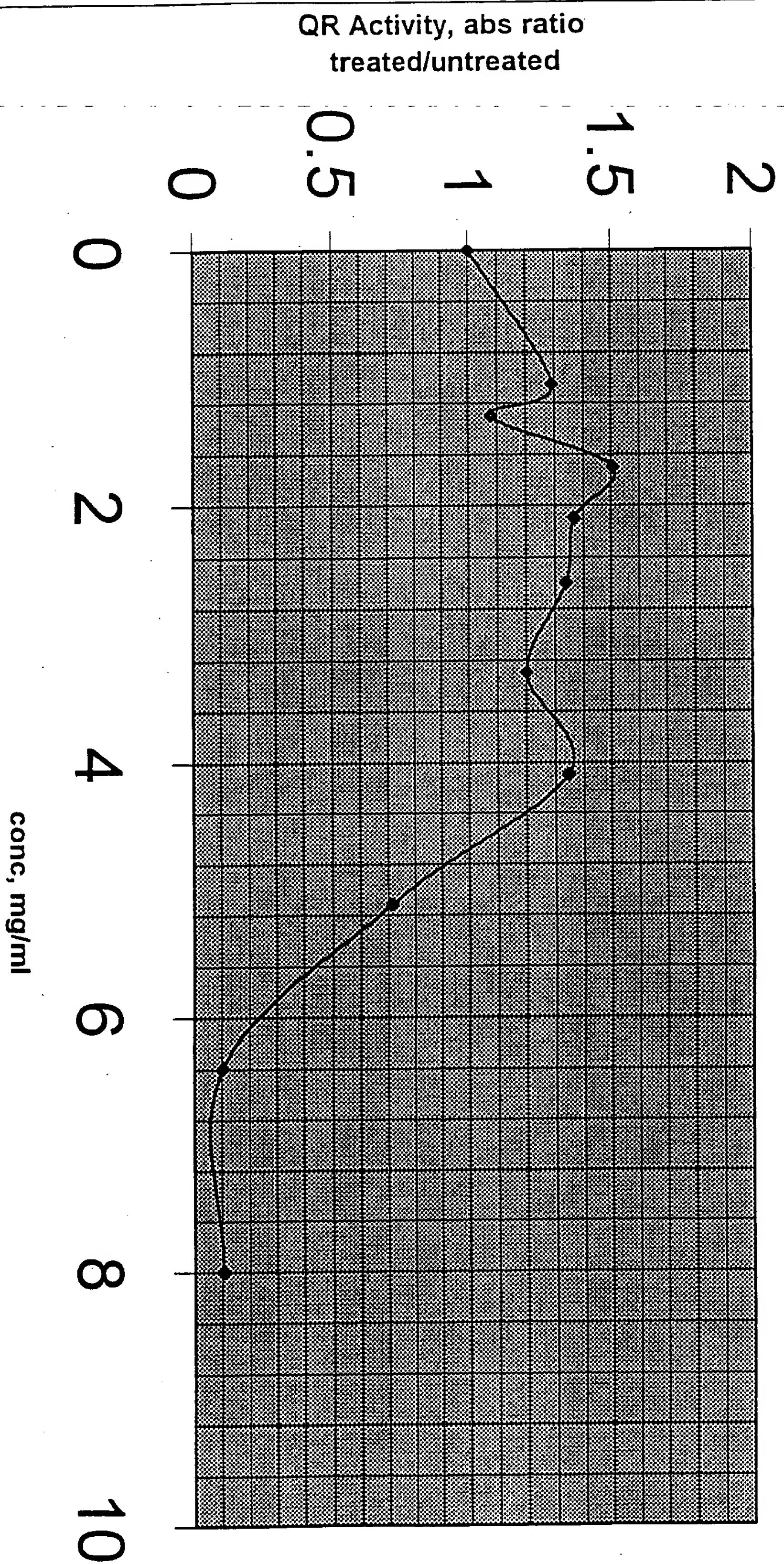
100 g *Echinacea purpurea* ground
roots or aerial parts (100 g)



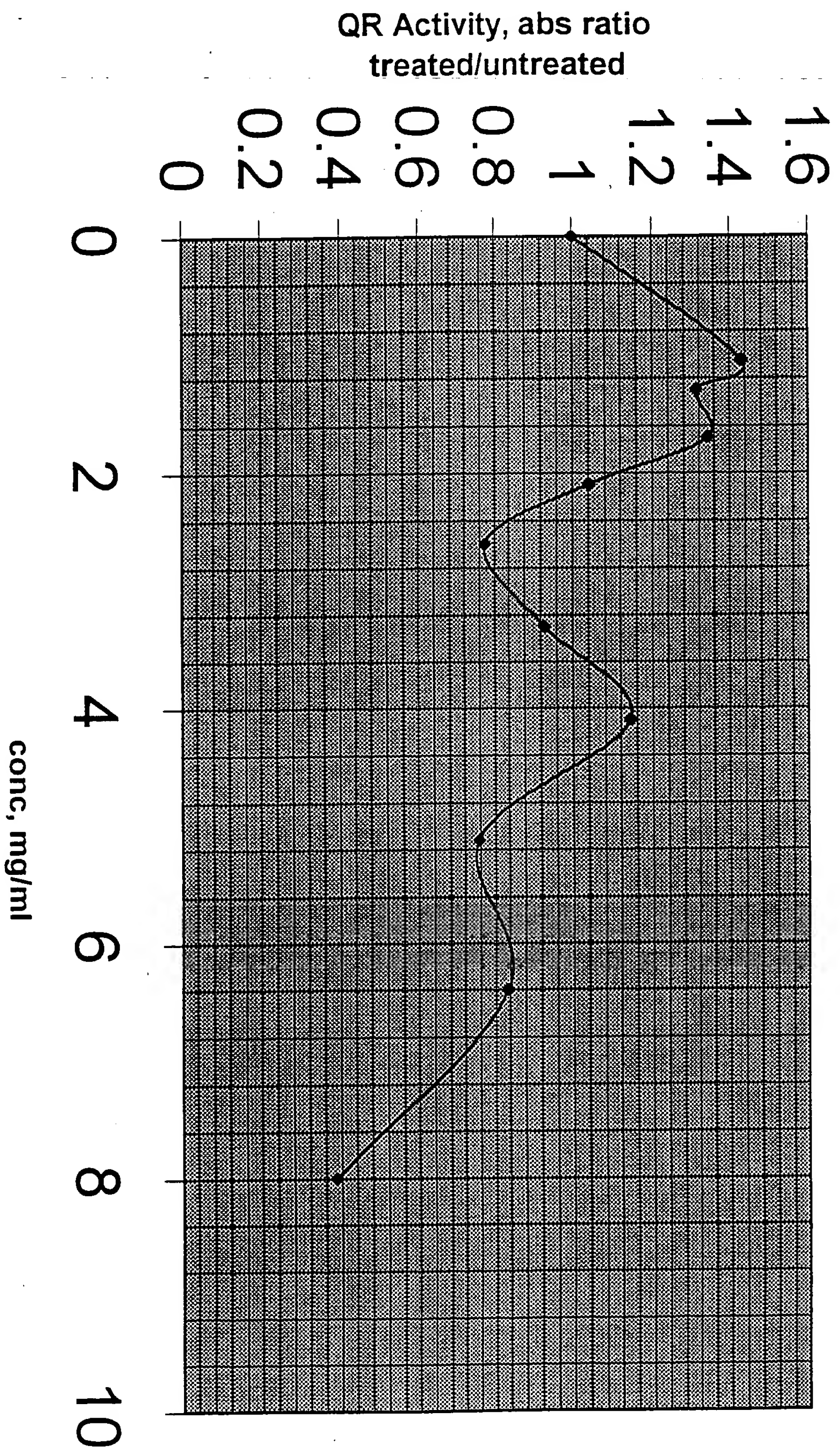
Echinacea purpurea root extract,
60 C extraction



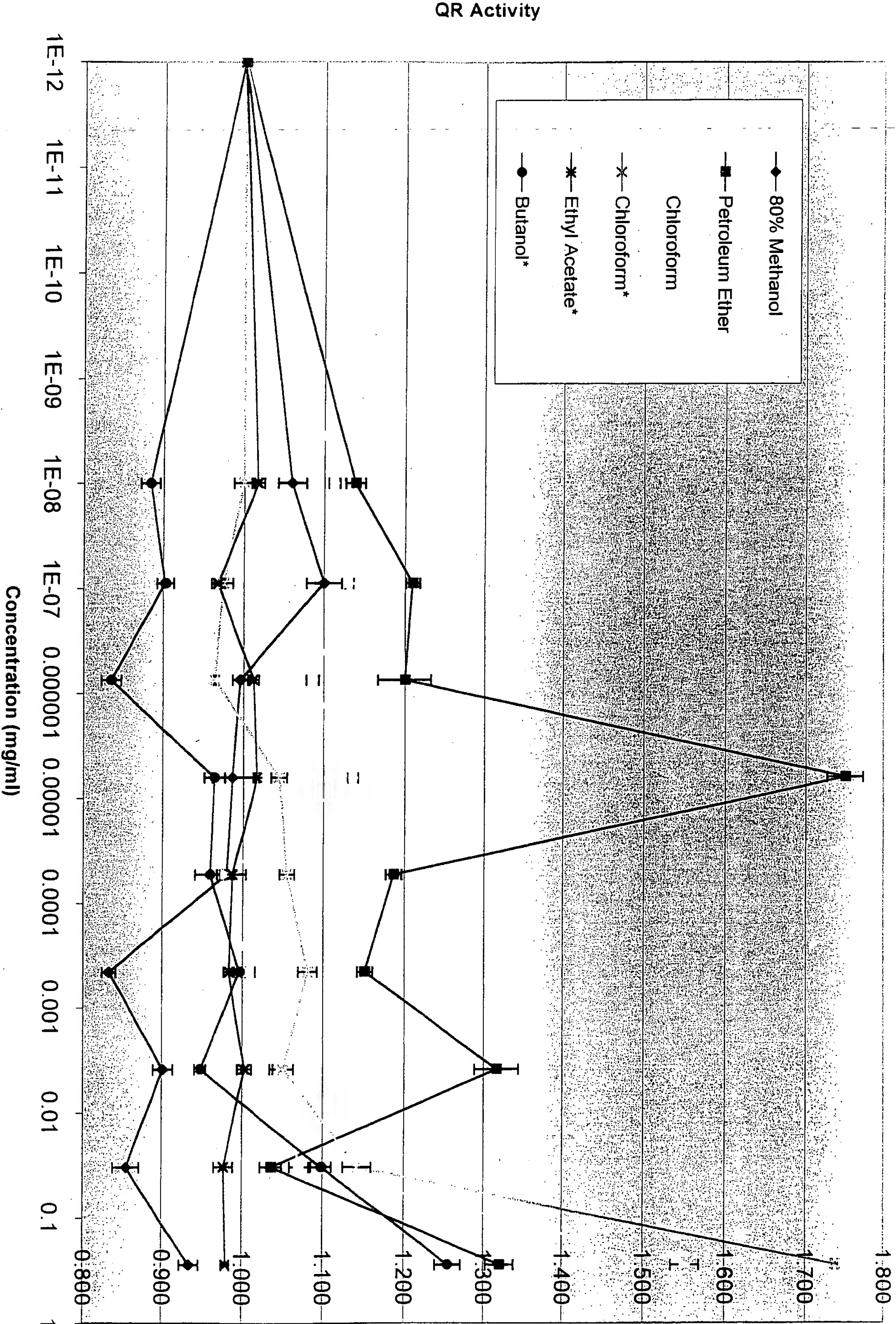
Echinacea purpurea root extract ,
20 C extraction



Echinacea purpurea aerial parts extract

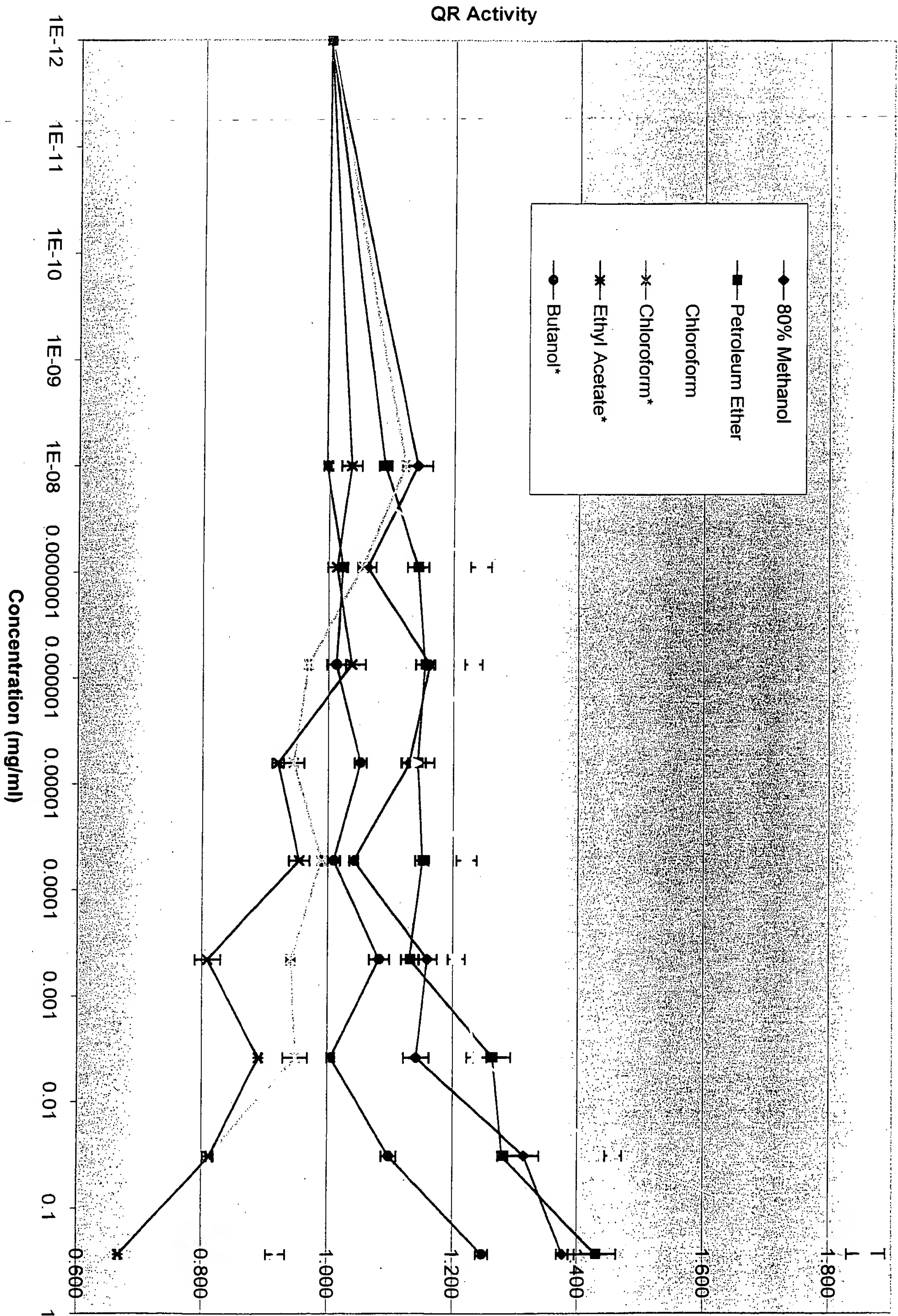


Induction of Quinone Reductase by *Echinacea purpurea* Aerial Fractions



* The aqueous residue was acidified to pH 2.

Induction of Quinone Reductase by *Echinacea purpurea* Root Fractions



* The aqueous residue was acidified to pH 2.

Protocol is from:

Maffei-Facino, R., Carini, M.,

(1993) *Echinacea* Extract

~~Caffoyl ester~~

100g Roots or Tops

by 80% MeOH twice

500ml x 2 times

① Condense 60min

② Condense 40min

Maffei-Facino, R., Carini, M.,

Aldini, G., et al. (1993)

Extract

Direct characterization of
caffoyl esters w/antihyaluronase
activity in crude extracts
from E. a. roots by fast atom
bombardment tandem mass
spectrometry. *Pharmacol* 48(10)
1947-1961

Dry

evaporate

@ 30-40°C

R=11.7g

T=20.5g

Residue

add 100ml H₂O + sonicate until
homogenous

Residue + H₂O

R=5.8g

T=10.2g

Fractionate (separatory funnel)

①

Petroleum Ether

100ml x 3

Na₂SO₄

evaporate

R=10.1g
1.72%
T=0.1584g
1.55%

②

Chloroform
Methyl Chloride

100ml x 3

Na₂SO₄

evaporate

R=0.2383g
4.11%
T=0.2185g
2.14%

③

Adjust pH
of H₂O to 2
using HCl

Fractionate

④

Methylene Chloride
(100ml x 3)

H₂O 50 x 2 wash

Na₂SO₄

evap

T=0.0442
R=0.10535
0.92%
0.43%
g

⑤

Ethyl Acetate
100ml x 3

H₂O 50 x 2 wash

Na₂SO₄

evap

R=0.0191
0.3295
10.67%
3.23%
g

⑥

Butanol
(100ml x 3)

H₂O 50 x 2 wash

Na₂SO₄

evap

R=0.0607g
0.0624g
1.05%
g

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Day 2: Tuesday

Prepare 2 MEM (w/ 10% calf serum + 1% antibiotic)

Prepare the samples:

#1 Brassica concentrate

$$100 \text{ mg sample} / 10 \text{ ml 2 MEM (w/calf serum + antibiotic)} \\ = \underline{10 \text{ mg/ml}}$$

#2 E. p. Roots

$$- 50 \text{ mg sample} / 10 \text{ ml 2 MEM (w/calf serum + antibiotic)} \\ = \underline{5 \text{ mg/ml}}$$

$$- 100 \text{ mg sample} / 10 \text{ ml 2 MEM (w/ " ")} \\ = \underline{10 \text{ mg/ml}}$$

#3 E. p. Tops

$$- 50 \text{ mg sample} / 10 \text{ ml 2 MEM (w/ " ")} \\ = \underline{5 \text{ mg/ml}}$$

$$- 100 \text{ mg sample} / 10 \text{ ml 2 MEM (w/ " ")} \\ = \underline{10 \text{ mg/ml}}$$

#4 E. p. Concentrate

$$50 \text{ mg sample} / 10 \text{ ml 2 MEM (w/ " ")} \\ = \underline{5 \text{ mg/ml}}$$

#5 E. p. Concentrate

$$100 \text{ mg sample} / 10 \text{ ml 2 MEM (w/ " ")} \\ = \underline{10 \text{ mg/ml}}$$

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- Vortex all of the samples for ≈ 2 minutes
- Then centrifuge @ speed 6 for ≈ 10 minutes
(IEC Clinical centrifuge)

Treatment w/ samples:

- 1.) Remove all of the old α MEM medium from the 96 well plates.
- 2.) To lanes 1 + 2 add 300ul α MEM medium, to lanes 3-12 add 150ul α MEM medium.
- 3.) Add 100ul sample to lane 12, pipet up + down 4 times, remove 100ul from lane 12 + put into lane 11, pipet up + down 4 times, remove 100ul from lane 11 + put into lane 10 pipet up + down 4 times... continue this through lane 3 @ lane 2 once all is mixed (4 times) remove 100ul + discard
- 4.) Repeat step #3 using 100ul of new sample.
- 5.) Add 150ul of α MEM medium to lanes 3-12, to make all wells have a final volume of 300ul.

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96 will state

controls



①

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6



100

3a. Add ^{and} 100 ml sample

① Add 150 μ l α MEM medium
to each lane 3-12

④ Make sure @ the end
that all wells have 300ul
total (Add 150ul MEM medium
to lanes 3-12 after treating w/
sample)

redacted

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Plate #1: Brassica Concentrate (10mg/ml)

923 brcc dat	controls	10mg/ml

- capture image

same as 92399br.cpf

Plate #2: E. purpurea Roots (5 + 10mg/ml)

923 eproots.dat	controls	5mg/ml
		10mg/ml

92399er.cpf

Plate #3: E. purpurea Tops (5 + 10mg/ml)

923 eptops.dat	controls	5mg/ml
		10mg/ml

92399et.cpf

Plate #4: E. purpurea Concentrate (5mg/ml)

923 epc50.dat	controls	5mg/ml

92399ec5.cpf

Plate #5: E. p. Concentrate (10mg/ml)

923 epc100	controls	10mg/ml

92399ec1.cpf

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Day 4 PII Enzyme Assay Reading

Day 3: Wednesday - I was sick, did not need to prepare cocktail, it was prepared last time + stored in freezer.

Day 4: Thursday - Reading + Evaluation of Results

- 1.) Look @ wells under microscope. Note if any of the concentrations caused cell death.
* do this b/4 decanting wells - adding Rxn cocktail

Plate #1: Brassica concentrate (10mg/ml)

Lane 9 - start to see dark cells - maybe dead
but when I move the plate they don't move
↓
keep seeing more + more through lane 12
Lane 12:

Plate #2: E. purpurea roots

5mg/ml

Lane 12 see some dark cells same as brassica concentrate

other lanes look fine

10mg/ml

Lane 9 start to see dark cells

↓
keep seeing more + more
Lane 12

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Plate #3

E. purpurea Tops

5mg/ml

lane 10 - start to see dark cells



lane 12 - ↑ dark cells

10mg/ml

lane 7 - start to see dark cells

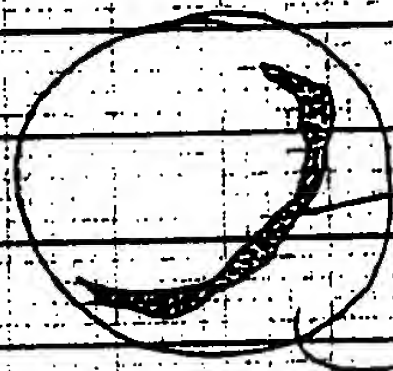


lane 12 - ↑ dark cells, still not that much

Plate #4

E. purpurea concentrate (5mg/ml)

- see a lot of the cells grouped together - dark brown -
- do not look attached but when you shake the wells
- they don't move, also see attached cells



cells brown (don't appear to be floating)

also see attached cells

Plate #5

E. purpurea concentrate (10mg/ml)

- same as plate appear the same as plate #4

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Cocktail - thaw, before assay add 100 units
glucose 6-phosphate dehydrogenase + 50 μ l
50mM Menadione in acetonitrile per 50ml cocktail
(previously prepared)

Enzyme Assay

- 1) Wash wells as completely as possible
- 2) Add 30 μ l of 0.8% digitonin in 2mM EDTA
(pH 7.4) to all wells + incubate 10min
@ 37°C to lyse cells. Then 10min
(@ 25°C) w/ 100rpm shaking (Orbital
shaker) to release enzymes
 \Rightarrow Orbital Shaker setting @ 4
- 3) Add 100 μ l cocktail + start timing
(2-10min) * 5 minutes is best time *
- 4) Stop the rxn by adding 30 μ l
of 0.3mM dicoumarol in 0.5%
DMSO + 5mM KH_2PO_4 (pH 7.4)
- 5) Read plate @ 410nm + record OD values
using microplate reader

Evaluate Data next week

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Cocktail: ~~show~~ before assay add 100 units glucose 6 phosphate dehydrogenase and 50ml of 50mM Mannitol in alcohol buffer
 => per 50ml aliquot of previously prepared cocktail

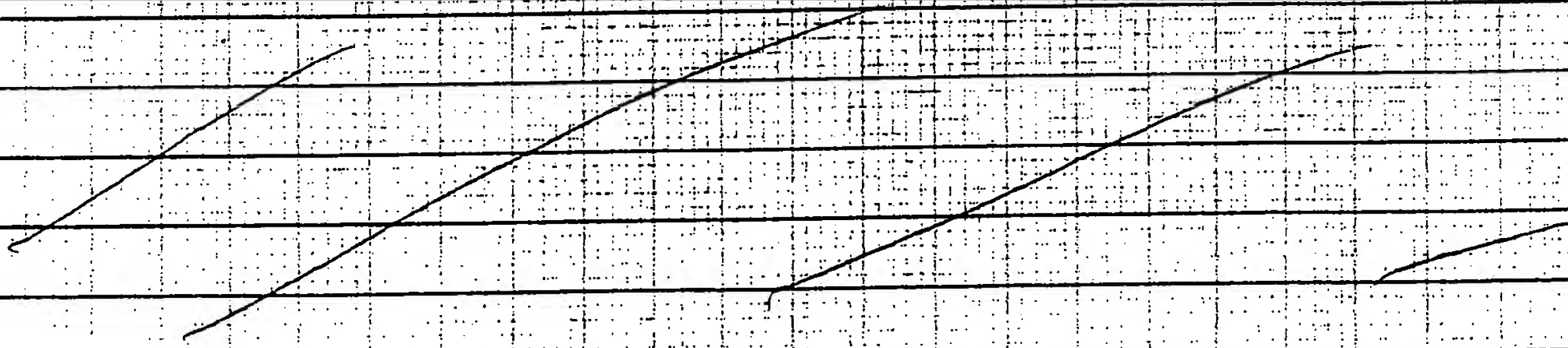
Enzyme Assay

Follow the steps on pg 117

Evaluate Data:

Record Appearance of water

See notebook # 7104



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#9 Chloroform pH.8 Roots (20mg/ml)

10799.6R2

Control:

Mean OD = 0.671

Std Dev = 0.043

CN = 6.342

20ul 3.65e-11 5.44e-10 8.12e-9 1.21e-7 1.81e-6 2.7e-5 4.03e-4 0.006 0.0898 1.34

Conc (mg/ml) 2e-4 2e-8 2e-7 2e-6 2e-5 2e-4 2e-3 2e-2 0.2 2

Mean OD 0.753 0.707 0.650 0.635 0.664 0.632 0.637 0.544 0.617 0.242

Std Dev 0.020 0.031 0.014 0.064 0.004 0.026 0.078 0.037 0.060 0.084

CN 2.620 4.334 2.165 10.10 0.643 4.165 12.25 6.726 9.754 34.85

QR Activity 1.122 1.054 0.969 0.946 0.990 0.942 0.949 0.811 0.994 0.361
0.920

20ul 2e-9 2e-8 2e-7 2e-6 2e-5 2e-4 2e-3 2e-2 0.2 2

Conc (mg/ml) 3.65e-11 5.44e-10 8.12e-9 1.21e-7 1.81e-6 2.7e-5 4.03e-4 0.006 0.0898 1.34

Mean OD 0.663 0.635 0.591 0.595 0.627 0.557 0.602 0.611 0.606 0.275

Std Dev 0.064 0.051 0.077 0.038 0.064 0.040 0.041 0.042 0.049 0.035

CN 9.596 8.111 13.40 6.353 10.27 7.252 6.877 6.912 8.162 12.79

QR Activity 0.988 0.946 0.851 0.887 0.934 0.830 0.897 0.911 0.903 0.410

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#10 Chloroform pHZ Tops (20mg/ml)

10799 ct 2

Control:

Mean OD = 0.610

Std Dev = 0.049

CV = 8.081

20ul	3.65e-11	5.44e-10	8.12e-9	1.21e-7	1.81e-6	2.7e-5	4.03e-4	0.006	0.0898	1.34
Conc (mg/ml)	2e-9	2e-8	2e-7	2e-6	2e-5	2e-4	2e-3	2e-2	2e-1	2
Mean OD	0.610	0.595	0.588	0.638	0.644	0.660	0.642	0.697	1.061	0.286
Std Dev	0.048	0.049	0.006	0.040	0.038	0.046	0.050	0.070	0.014	0.039
CV	7.807	8.234	0.935	6.338	5.932	7.030	7.798	10.05	1.282	13.77
GR Activity	1.0	0.975	0.964	1.046	1.056	1.082	1.052	1.143	1.739	0.469

30ul	2e-9	2e-8	2e-7	2e-6	2e-5	2e-4	2e-3	2e-2	0.2	2
Conc (mg/ml)	3.65e-11	5.44e-10	8.12e-9	1.21e-7	1.81e-6	2.7e-5	4.03e-4	0.00602	0.0898	1.34
Mean OD	0.611	0.642	0.618	0.638	0.623	0.679	0.712	0.737	0.961	0.380
Std Dev	0.047	0.049	0.021	0.026	0.058	0.030	0.062	0.038	0.101	0.006
CV	7.733	7.620	3.357	4.110	9.312	4.484	8.648	5.196	10.49	1.493
GR Activity	1.002	1.052	1.013	1.046	1.021	1.113	1.167	1.208	1.575	0.623

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